Impact of free-living diazotrophs, *Azospirillum lipoferum* and *Gluconacetobacter azotocaptans*, on growth and nitrogen utilization by wheat (*Triticum aestivum* cv. Lillian)

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ABSTRACT

Nitrogen (N) is an essential plant nutrient, widely applied as N-fertilizer to improve yields of agriculturally important crops. An alternative to fertilizer use could be the exploitation of plant growth-promoting bacteria, capable of enhancing growth and yield of many plant species. *Azospirillum* and *Gluconacetobacter* are root colonizing, free-living, N_2-fixing bacteria (diazotrophs) with the potential to transfer fixed N to associated plants.

The purpose of this study was to evaluate the agronomic efficiency of two diazotrophs, *Azospirillum lipoferum* and *Gluconacetobacter azotocaptans,* inoculated onto wheat. Physiological parameters and yield components were evaluated. The objectives of this study were to: 1) determine the survival of each diazotroph species on wheat seeds over time; 2) determine the survival of *A. lipoferum* and *G. azotocaptans* inoculated on wheat seed treated with a fungicide seed treatment, Dividend® XL RTA®; 3) determine if inoculation of wheat with the diazotrophs under controlled conditions causes an increase in dry matter, N_2-fixation and N uptake; 4) determine if fertilizer N applied at three levels influences atmospheric N_2-fixation by *A. lipoferum* or *G. azotocaptans*; 5) determine if inoculation of wheat with *A. lipoferum* or *G. azotocaptans* under field conditions causes any increase in dry matter, N_2-fixation and N uptake; 6) determine if N-fertilization levels under field conditions influenced N_2-fixation by *A. lipoferum* or *G. azotocaptans.* In order to meet these objectives lab, growth chamber, and field studies were completed.

Laboratory investigations revealed that the decline in recovery of colony forming units (CFU) of *G. azotocaptans* was not significantly different ($P<0.05$) for any seed treatment. There was a general decrease in CFU over time regardless of seed treatment. Analysis of the recovered CFU of *A. lipoferum* over time showed that there was a significant difference ($P<0.05$) between both the non-sterilized seed and the Dividend® XL RTA® treated seed when compared sterilized seed. Recovery of CFU on sterilized seed declined at a more rapid rate compared to the other two seed treatments. *Gluconacetobacter azotocaptans* and *A. lipoferum* were not negatively influenced by the Dividend® XL RTA® seed treatment. Also, both diazotrophs were able to compete with other microorganisms that may have been on the seed coat of unsterilized seeds.

Azospirillum lipoferum and *G. azotocaptans* were able to fix atmospheric N, but, there were no significant (P<0.05) differences between the diazotroph species. Additions of fertilizer N enhanced N_2-fixation, in both the growth chamber and field studies. As the amount of fertilizer N increased, so did the %Ndfa and N uptake. In the growth chamber study, inoculated wheat, and fertilized with 12.2 and 24.5 μg N g^{-1} had the highest %Ndfa of 25.5%, and wheat fertilized with 24 μg N g^{-1} had the highest N uptake (1.3 g pot^{-1}) at maturity. In the field study, inoculated wheat fertilized with of 80 kg N ha^{-1} had significantly higher (P<0.05) %Ndfa (10.5%) compared to wheat grown with the other fertilizer levels, which also corresponded to the highest N uptake in wheat plants (47 kg ha^{-1}).

The diazotrophs also affected the partitioning of N in the wheat plants differently. Wheat inoculated with *A. lipoferum* had significantly higher (P<0.05) amounts of N accumulated in heads of plants, and wheat inoculated with *G. azotocaptans* had significantly higher (P<0.05) amounts of N accumulated in stems of plants. However, this trend was not evident in the field study.

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DEDICATION

For my mom and dad for your continuous love and support.

And to my Grandparents for always asking if I was finished yet…

You knew I could do it!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

%Ndfa = percent nitrogen derived from the atmosphere
ANOVA = analysis of variance
Azo = *Azospirillum lipoferum*
BNF = biological nitrogen fixation
CFU = colony forming units
Cont = control
dia = diameter
fert = fertilizer
Gluc = *Gluconacetobacter azotocaptans*
h = hour
ha = hectare
loc = location
MHC = moisture holding capacity
min = minute
NSNF = non-symbiotic nitrogen fixation
PGPB = plant growth promoting bacteria
PGPR = plant growth promoting rhizobacterium
rep = replicate
RO = reverse osmosis
trt = treatment
wk = week

1. INTRODUCTION

This research aims to evaluate our current understanding about non-symbiotic nitrogen (N_2) fixation in agriculture systems and to explore the potential to increase this source of N through inoculation with bacteria. Non-symbiotic N_2-fixation includes N_2-fixation by free-living bacteria (autotrophic and heterotrophic) that are not in a direct symbiosis with plants. Non-symbiotic N_2-fixation also includes associative N_2-fixing systems (e.g., in grasses and cereals), cyanobacteria in symbioses with lichens and bryophytes, and N_2-fixing actinobacteria associated with non-leguminous plants (Unkovich and Baldock, 2008).

Nitrogen is an essential plant nutrient, and is the most commonly-deficient nutrient, contributing to reduced agricultural yields throughout the world (Saikia and Jain, 2007). It is a major component of dietary proteins, but plant uptake efficiency is low and large amounts can be lost to denitrification and leaching. Globally, roughly 83 million tonnes of N fertilizers are produced each year by the Haber-Bosch process (Jenkinson, 2001). However, this process uses large amounts of fossil fuel and requires high capital and energy costs (van Berkum and Bohlool, 1980; Jensen and Hauggaard-Nielsen, 2003). Unfortunately, less than 50% of applied N fertilizer is used by plants (Garabet et al., 1998; Halvorson et al., 2002; Saikia and Jain, 2007). With increasing costs of chemical fertilizers, concern about environmental pollution, and increasing demand for organically-grown agricultural and horticultural products has rekindled interest in promoting biological nitrogen fixation (BNF) as a biofertilizer to meet crop N-requirements (Vessey, 2003).

A common theme of most research is the relationship between BNF and sustainable agriculture. Almost by definition, BNF is synonymous with sustainability (Saikia and Jain, 2007). Systems capable of fixing their own N exploit their own environment less and many even provide a positive N contribution. Cleveland et al. (1999) estimated that the potential global BNF (symbiotic and non-symbiotic) in natural ecosystems is between 100 and 290 million tonnes N $year^{-1}$. Symbiotic N_2-fixation is the dominant form and the estimates are higher for the tropics than for more temperate regions. However, there are critical gaps in the knowledge of BNF and particularly non-symbiotic N_2-fixation.

Most estimates of non-symbiotic N_2-fixation have been determined by indirect measures such as acetylene (C_2H_2) reduction or a calculation from N balances (Unkovich and Baldock, 2008). Reports on grass associations claim contributions of N via non-symbiotic N_2-fixation of 20 to 50 kg N ha^{-1} $year^{-1}$ (Parker, 1957; Greenland, 1971; Chaulk, 1991) with reports as high as 100 kg N ha^{-1} $year^{-1}$ in tropical grasses (Weier, 1980; Weier et al., 1995). Field measurement of N_2-fixation by free-living bacteria using crop residues as an energy source indicated 1 to 12 kg N ha^{-1} fixed during short periods of 2 to 4 wk (Roper, 1983; Roper et al., 1989; 1994), but where warm, moist conditions coincide with fresh stubble, annual potentials of up to 38 kg N ha^{-1} $year^{-1}$ have been calculated (Gupta et al., 2006). These rates for free-living N_2-fixation compare well with ranges of 0 to 60 kg N ha^{-1} $year^{-1}$ measured elsewhere and reviewed by Bürgmann et al. (2004). Rates of non-symbiotic N_2-fixation are significantly less than estimated N inputs from symbiotic N_2-fixation which range from 2 to 284 kg N ha^{-1} $year^{-1}$ in legume pastures (Peoples and Baldock, 2001), and 0 to 271 kg N ha^{-1} $year^{-1}$ in grain legumes (Unkovich et al., 1994; Evans et al., 2001). Nonetheless, non-symbiotic N_2-fixation has the potential to contribute significantly to the N requirements of a crop.

It takes roughly 26 kg N to produce 1 tonne of wheat grain, including straw (Bhuiyan, 1995; Angus, 2001; Kennedy et al., 2004), but at peak demand in a growing crop, N demand exceeds supply from N mineralization (Angus, 2001). Currently, N fertilizers compensate for much of this shortfall. If biological systems can be manipulated to increase the inputs of N from non-symbiotic N_2-fixation it should be possible to reduce the requirement for increasingly expensive industrially-fixed N fertilizers.

Many of the measurements of non-symbiotic N_2-fixation were made more than 30 years ago. Since then, there have been significant changes in our farming systems around the world. Some of the key changes in farming systems have been a move toward intensive cropping systems, no-tillage and stubble retention, and growing genetically mondified crops. All of these changes are likely to have significant impacts on non-symbiotic N_2-fixation through the provision of larger carbon (C) resources for biological activity, and the creation and preservation of ideal conditions for non-symbiotic N_2-fixation. Therefore, it is important to re-evaluate biological systems in the light of these factors.

The objective of this study was to evaluate the effects of two non-symbiotic N_2-fixing bacteria (diazotrophs), *Gluconacetobacter azotocaptans* and *Azospirillum lipoferum,* on N acquisition by wheat. This was done by testing if the diazotroph strains caused an increase in N uptake and dry matter over an uninoculated treatment, if one diazotroph strain increased N uptake and dry matter in wheat over the other, and by understanding how long the diazotroph strains survive on wheat seeds after inoculation.

2. LITERATURE REVIEW

2.1 Nitrogen fixing (diazotrophic) populations

It is now well over 100 years since the existence of microorganisms capable of biological fixation of atmospheric nitrogen (N) was experimentally proven (Tchan, 1988). Barely 100 years ago, the N_2-fixing capacity of the legume-rhizobial symbiosis was firmly established (Mia and Shamsuddin, 2009). Since then, this symbiotic system has become well understood and exploited as an effective means of raising the N status of soils, and providing N for crops and pastures (Vincent, 1984). Optimism that non-leguminous crops could benefit similarly was fueled in the 1970's and 1980's by the discovery in the 1950's of several N_2-fixing organisms, diazotrophs (Döbereiner, 1953; Döbereiner and Ruschel, 1958; Ďobereiner, 1966; Kennedy and Tchan, 1992). These diazotrophs formed apparently specific associations with non-legumes.

Since then, Dalton (1980) and Roper and Ladha (1995) were able to identify more than 50 different genera of culturable diazotrophic bacteria. More recently, new molecular technologies utilizing analysis of the *nif*H gene, a structural gene for the highly conserved nitrogenase protein, and stable isotope (^{15}N) probing have identified a suite of previously unrecognized diazotrophic microorganisms (e.g., Hamelin et al., 2002; Bürgmann et al., 2004; Buckley et al., 2007; Roesch et al., 2008). Buckley et al. (2007) cited a series of studies that suggested these non-culturable diazotrophs may be the dominant N_2-fixing microorganisms in soils compared with their culturable cousins. The capacity for non-symbiotic N_2-fixation is extensive across bacteria and Archaea and is performed by chemotrophs, phototrophs and heterotrophs (Bürgmann et al., 2004). This great diversity ensures the adaptability of populations of N_2-fixing microorganisms to a wide range of conditions. This is reflected in the studies by Zhang et al. (2007) and Bürgmann et al. (2004) who observed that at any one time, the actively N_2-fixing population represented only a very small subset of the total diazotrophic population.

Some non-symbiotic N_2-fixing bacteria have been found as endophytes, both obligate and facultative (e.g., Baldani et al., 1993, Baldani et al., 1997; James, 2000; Hurek et al., 2002; Cocking, 2003; Roesch et al., 2008). Prior to their discovery the only endophytic N_2-fixing bacteria recognized were those in legume-*Rhizobium* or Casuarinas-Frankia symbiosis. The term

endophyte was first introduced to the area of N_2-fixation research associated with Graminaceous plants by Döbereiner (1992a, b). In general, the term includes all microorganisms that are able to colonize, during some portion of their life cycle, the inner tissues of plants without causing any apparent damage to the host (Petrini, 1991). Endophytic bacteria are at an advantage compared with free-living or rhizosphere bacteria because they have ready access to nutrients and water from the plant (Wilson, 1995), and are not as vulnerable to competition from other microorganisms in the rhizosphere or soil. The plant interior may also provide an environment conductive to N_2-fixation by being low in oxygen (O_2) and relatively high in carbon (C). The bacteria can fix N_2 more efficiently in association with a plant host (James and Olivares, 1998).

Endophytic organisms are more likely to be successful as inoculants. As with non-endophytic diazotrophs, non-culturable N_2-fixing microorganisms appear to be dominant. Hurek et al. (2002) used polymerase chain reaction-amplified *nifH* transcripts to detect and quantify nitrogenase activity by an N_2-fixing bacterial endophyte, *Azoarcus* spp., which was active in an unculturable state in kallar grass (*Leptochloa fusca).* From their phylogenetic analysis of nitrogenase sequences, they predicted that uncultured grass endophytes are ecologically dominant and could play an important role in N_2-fixation in natural grass ecosystems. Furthermore, they showed that culturable diazotrophs were neither abundant nor active and probably do not contribute significant N in associations with the Gramineae family. Use of new molecular technologies should expand our knowledge of this apparently large group of non-culturable but active N_2-fixing bacteria.

2.1.1 Non-leguminous N_2-fixing systems

Attempts to discover beneficial associations between plants and N_2-fixing bacteria have a long history. Obviously, a large portion of the total microbial activity in soil is likely to be associated with the growth of plants, or their subsequent breakdown, simply because this is where growth substrates occur in the greatest abundance. For N_2-fixers, Döbereiner (1989) stressed that too much may be assumed about the degree of an associative relationship simply because bacteria are located in the rhizosphere near the root surface. It may also be remembered that as long as the N_2-fixation carried out by such diazotrophs remains bound to the growth of the bacterium, the possibility of a significant effect on the plant remains slight.

2.1.1.1 *Azotobacter*

Azotobacter spp. are aerobic, free-living soil microbes that fix N from the atmosphere. Besides N_2-fixation, *Azotobacter*, when applied to seeds, improves seed germination to a considerable extent (Soleimanzadeh et al., 2010). *Azotobacter* will also control plant diseases due to substances it produces. This was one of the organisms involved in early research, with favorable results reported from Russia by Kostychev et al. (1926), though the findings in Russia and elsewhere were generally inconsistent and variable (Mishustin and Shil'Nikova, 1971). The problem of inconsistency has not been overcome by using well authenticated strains of inoculum. One of the problems with *Azotobacter* is its poor performance in colonizing the rhizosphere. Also, several processes other than N_2-fixation could have accounted for any positive effects in field trials, including production of growth regulators, protection from root pathogens, and modification of nutrient uptake by the plant (Tchan, 1988).

2.1.1.2 *Clostridium*

Sensitivity to molecular O_2 generally restricts *Clostridium* spp. to anaerobic areas such as water and submerged soil (Cato et al., 1986). Anoxic microsites existing in soil particles (Sexstone et al., 1985) and litter (van der Lee et al., 1999) often provide a habitat for clostridia because they have been isolated from non-submerged soil and litter (Kuhner et al., 2000). Early work has suggested the presence of N_2-fixation by strictly anaerobic organisms and clostridia in the rice rhizosphere (Hirota et al., 1978) and by the soil microbial community in aerobic cultures (Line and Loutit, 1973). Therefore, it is not surprising that clostridia reside in the aerial parts of plant tissues, which are exposed to the air and to O_2 produced by photosynthesis. The plant-dwelling clostridia probably sometimes proliferate in anoxic microzones produced by anaerobic N_2-fixing consortiums or plant respiration, while they survive in spore forms under higher O_2 concentrations (Minamisawa et al., 2003).

Species such as *Clostridium pasteurianum* and *Cl. butyricum* are strict anaerobes, and therefore fix N only in the absence of O_2 (Saralov and Babanazarov, 1983; Kennedy and Tchan, 1992). Provided that sufficient soluble energy substrates are available, *Clostridium* spp. grow readily and are quite easy to isolate in N-deficient media using soil as inoculum.

2.1.1.3 Photosynthetic N_2-fixing bacteria and cyanobacteria

Cyanobacteria, formerly known as blue-green algae, are a phylum of bacteria that obtain their energy through photosynthesis. They are a significant component of the marine N cycle, but are found in almost every conceivable environment on earth. The metabolic process of N_2-fixation is expressed under anaerobic, microaerobic or aerobic growth conditions by unicellular and filamentous species (Rippka et al., 1979). In the heterocyst-forming species, nitrogenase expression appears to be confined to the heterocysts (Elhai and Wolk, 1990), whereas it may be active in any or all cells of the non-heterocyst forming species (Meeks et al., 1994).

2.1.2 Associative diazotrophic systems

Attempts to discover beneficial associations between plants and N_2-fixing bacteria have a long history. Obviously, a large portion of the total microbial activity in soil is likely to be associated with the growth of plants, or their subsequent breakdown, simply because this is where growth substrates occur in the greatest abundance. It may be remembered that as long as the N_2-fixation carried out by such diazotrophs remains bound to the growth of the bacterium, the possibility of a significant effect on the plant remains slight. Nevertheless, certain bacterial species (in particular the genus *Azospirillum)* are thought to have a close association with plants, to a point bordering on symbiosis.

2.1.2.1 *Azospirillum*

Azospirillum are free-living bacteria, some of which are known to be plant-growth-promoting bacteria (PGPB), capable of affecting growth and yield of numerous plant species (Mehnaz and Lazarovits, 2006). This organism is known to penetrate the root of gramineous plant species and to grow intercellularly to a degree (Sumner, 1990; Baldani et al., 1993), as well as growing in the rhizosphere (Reinhold and Hurek, 1989). Plant-growth promotion by *Azospirillum* is not fully understood. Initially, it was known only as a N_2-fixer, but the current opinion is that the primary mechanism is related to production of growth-promoting substances such as cytokinins, gibberellins, and auxins (Zimmer, 1988; Frankenberger and Arshad, 1995; Somers and Vanderleyden, 2004).

Azospirillum strains are routinely isolated from agricultural lands and crop plants, including traditional isolations from grasses and cereals (Nath et al., 1997; Weber et al., 1999). Although some of their host species are perennials, most are annuals (Bashan and Holguin, 1997a). This creates a hurdle for survival from one season to the next, especially when harsh (both high and low) temperatures combined with a long period of drought prevail.

Although microorganisms in general and *Azospirillum* spp. in particular, have shown great promise in *in vitro* screening, the expression of their beneficial properties in the natural environment is unpredictable and often disappointing (Bashan and Levanony, 1990; van Elsas and Heijnan, 1990; Bashan, 1993; Kenney, 1997; Stotzky, 1997; Dobbelaere et al., 2001). In some of those trials *Azospirillum* was used in combination with other microorganisms, including other PGPB, arbuscular mycorrhizal fungi, rhizobia and microalgae (e.g., Gonzalez and Bashan, 2000; Dardanelli et al., 2008).

2.2 Measurement/Quantification

Quantities of fixed N vary depending on the N_2-fixing system in question, but in general free-living and associative N_2-fixers would fix much less N than symbiotic systems where host plants directly supply the micro-symbiont with energy and protect the nitrogenase enzyme from deactivation by O_2 (Bergersen, 1991).

Current methods of quantifying non-symbiotic N_2-fixation are far from perfect and measurement may be flawed if inappropriate techniques or inadequate controls are used. The most prominent flaws are from methodological problems. The low activities provided by free living bacteria (Knowles, 1980), the uncertainties of the application of the C_2H_2 reduction assay of soils (Lethbridge et al., 1982), the insensitivity of the $^{15}N_2$-fixation method (Bergersen, 1980), the great variability of nitrogenase in soil cores (Nohrstedt, 1985) and the complexity of such systems are some of the restraints inherent to this kind of investigation. However, used appropriately, these techniques may still provide some valuable insight into the role and importance of non-symbiotic N_2-fixation. New molecular technologies promise further advances in our ability to evaluate non-symbiotic N_2-fixation in soils and this will be addressed later in this review.

Aside from the difficulties and errors, the demand for accurate determinations of global inputs of biologically-fixed N is strong and will continue to be fuelled by the need to understand and effectively manage the global N cycle.

2.2.1 C_2H_2 reduction assay

The C_2H_2 reduction assay is a highly-sensitive assay based on the reduction of C_2H_2 to C_2H_4 by nitrogenase, which is universally-responsible for biological N_2-fixation. Nitrogenase is the only enzyme known to carry out this reduction (Witty, 1979). The assay was evaluated in detail by Hardy et al. (1968), and they found a direct correlation between N_2-fixation ($N_2 \longrightarrow 2NH_3$) and $C_2H_2 \longrightarrow C_2H_4$ in pure cultures of diazotrophs and in legumes with a quantitative relationship of C_2H_2 reduced to N_2 fixed of between 3 and 4. Witty (1979) also found that the assay accurately predicted N_2-fixation in pure cultures, but in soils either alone or containing pasture grasses, he observed an overestimation of the amount of N_2-fixation in the soil because C_2H_2 prevented the oxidation of natural C_2H_4 produced in the soil. To overcome this, simple controls with small concentrations of C_2H_2, sufficient to repress natural C_2H_4 production but insufficient for C_2H_2 reduction, were suggested by Nohrstedt (1983).

Giller (1987) and Giller and Merckx (2003) have been particularly critical of the use of the C_2H_2 reduction assay for the measurement of associative N_2-fixation in cereals and grasses. This was primarily because of the overestimation of N_2 and C_2H_2 prevention of oxidation of natural C_2H_4 produced in soil, which were identified by Witty (1979), and also due to long incubation times used in assays resulting in oxygen starvation of roots and changes in microbial behavior. The use of appropriate controls together with $^{15}N_2$ gas exposure for absolute measurement of N_2-fixation and calibration of the C_2H_2 reduction assay were strongly recommended (Giller, 1987).

A general criticism of the C_2H_2 reduction assay is that ratios of C_2H_4 produced to N_2 fixed can differ from the theoretical ratio of 3:1 (Unkovich and Baldock, 2008). Hardy et al. (1973), in a review on the applications of the C_2H_2 reduction assay, summarized published values of the C_2H_2/N_2 conversion factors available at the time. They found that for all systems including pure cultures, legumes, non-legumes and soils that this ratio averaged between 2.6 and 6.9. The only exception to this was anaerobic soil which had a conversion factor of up to 25.

This indicates that the use of the theoretical conversion factor under anaerobic conditions will greatly overestimate the amount of N_2 fixed. However, in other conditions a reasonable estimate is possible although any experimental procedure should always include calibration of the assay using $^{15}N_2$ gas exposure (Steyn and Delwiche, 1970).

Acetylene in soils can affect processes other than N_2-fixation, including nitrification and denitrification, and ethylene oxidation is reduced in the presence of C_2H_2, independent of N_2-fixation (Witty, 1979), all of which can have an impact on apparent nitrogenase activity (Giller and Wilson, 1993). Endogenous ethylene accumulation can be accounted for, to some extent, by the use of careful controls to measure background ethylene production (Roper, 1983), although again, this is not routinely done in all studies. A final consideration is high soil water contents, which are usual in assay systems to decrease O_2 availability and therefore increase nitrogenase activity. This can cause saturation of nitrogenase with C_2H_2, which does not occur with N due to a much lower diffusivity of N in soil water than C_2H_2 (Knowles, 1980). It has been found to then lead to overestimates of N_2-fixation under high water contents, especially in finer textured soils (Dart, 1986). Saturation of nitrogenase with C_2H_2 also gives apparently higher nitrogenase activity as this decreases the simultaneous hydrogen (H_2) production by nitrogenase.

Due to the sensitivity of the nitrogenase enzyme to O_2 (Eady, 1980), N_2-fixation in soils may only occur at microsites of low O_2 availability particularly in soils with moderate soil moisture contents. C_2H_2 is very soluble in water (42.8 mM at 1 atom% and 20°C; Turner and Gibson, 1980) and this may facilitate a rapid uptake of C_2H_2 at the sites of fixation and hence enable short assay times for measuring free-living N_2-fixation, thus reducing the possibility of negative impacts described above.

The C_2H_2 reduction assay is a rapid, simple, low cost method which if used with appropriate controls and calibrations can be useful for evaluating nitrogenase activity in time and space. Under controlled conditions it can be extremely useful for comparative purposes where absolute values of N_2-fixation are not critical.

2.2.2 $^{15}N_2$ gas as a direct measure of N_2-fixation

An early report on the use of $^{15}N_2$ gas to confirm N_2-fixation was provided by Burris et al. (1943). Historically, the availability of materials enriched with ^{15}N and mass spectrometers to

analyze the samples severely restricted its general application. However, this method of measurement has been in use for many years. This method can provide absolute proof of N_2-fixation and has been used to demonstrate N_2-fixation associated with cereals and grasses (Witty and Day, 1978; Giller et al., 1988), and in soils (Witty and Day, 1978; Roper, 1983; Azam et al., 1988). The method can be sensitive and accurate, provided the $^{15}N_2$ gas is free of contaminants including $^{15}NH_3$. Bergersen (1980) described a method for the production of 'clean' $^{15}N_2$ gas for use in such experiments. The use of $^{15}N_2$ on a large scale is limited by its costs, but it is a most useful method for calibrating other measures of N_2-fixation. Difficulties in controlling environmental conditions can be encountered when using the method to measure N_2-fixation associated with plants. Witty and Day (1978) devised an elaborate system for controlling the conditions around a growing plant during exposure to $^{15}N_2$.

An experiment by Giller et al. (1984) was able to show a clear demonstration that detectable amounts of N were fixed by bacteria in growth medium using a chamber for incubation of intact root systems and growth medium in $^{15}N_2$ enriched gas mixtures. They also established that fixed N was incorporated into the plant roots and shoots within 3 days of initial exposure to the gas. After a further week of growth, there was a higher enrichment of ^{15}N in the roots and the enrichment in the shoots had almost doubled (Giller et al., 1984).

Demonstrating the incorporation of $^{15}N_2$ into free-living microbial populations in the soil is much more difficult. Although N_2-fixation in soils was confirmed both in the laboratory and in the field by incorporation of $^{15}N_2$ (Alexander and Zuberer, 1989; Elbeltagy et al., 2001; Buckley et al., 2008), absolute measures of N_2-fixation in the field using $^{15}N_2$ gas were not possible. Many free-living, diazotrophic bacteria require reduced O_2 concentrations to fix N and are located within microsites of low O_2 tension. Sites that restrict O_2 availability may also limit access by $^{15}N_2$ and this could result in an underestimation of N_2-fixation.

2.2.3 ^{15}N isotope dilution

An alternative approach in using $^{15}N_2$ is to label soil with ^{15}N in the form of salts [e.g., $(^{15}NH_4)_2SO_4$ or urea] and observe dilution of this ^{15}N with atmospheric ^{14}N from BNF. Details of the isotope dilution method are described by Boddey et al. (1983), Chaulk (1985) and Boddey (1987). The isotope dilution technique involves supplying a ^{15}N enriched, or depleted, source of

N to the soil so that it is significantly different from the natural enrichment of the atmospheric N_2. The relative proportions of N derived from the air (via fixation) and from mineral sources in the soil can then be calculated.

Precautions in the use of the method primarily relate to the choice of the non-fixing control (reference plant) which should be closely related to the fixing plant and whose roots should explore the same volume of soil (Boddey et al., 1983). Chaulk (1985) also points out that the fixing and reference plants may not obtain the same proportion of labeled and unlabelled N from soil when the spatial and temporal availability of the isotope is not uniform. The largest source of error will be in the measurement of total N, which is required to calculate the amount of N_2-fixed, and the measurement of other N cycle fluxes such as N deposition, leaching and denitrification, could be the same order of magnitude as free-living N_2-fixation. However, the ability to measure small changes in the ^{15}N isotope composition of soils enables yield independent assessment of the fraction of soil total N that has come from N_2-fixation to be calculated (Unkovich and Baldock, 2008).

The ^{15}N isotope dilution method has been used to estimate N_2-fixation associated with sugarcane, forage grasses, cereals, and actinorhizal plants grown in soil, but mostly in tropical systems.

2.2.4 Natural abundance ($\delta^{15}N$)

The natural abundance ($\delta^{15}N$) method is exactly analogous to the isotope dilution method except that endogenous ^{15}N in the soil is used. The principles and details of the method are described by Shearer and Kohl (1988), Högberg (1997) and Boddey et al. (2000; 2001). The natural abundance method has an advantage over isotope methods in natural ecosystems because disturbance of the system is unnecessary (Shearer and Kohl, 1988) but, because there are variations in N derived from soil by different non-N_2-fixing plants, it is very difficult to distinguish between plants that are benefiting from N_2-fixation and those that are not (Boddey et al., 2001). Other factors that affect $\delta^{15}N$ in plants are sources of N other than from N_2-fixation or from the soil, such as from precipitation (NH_3), the depths in the soil from which N is taken up, and the form of soil N that is used (organic N, NH_4^+ or NO_3^-; Högberg, 1997). The ability of the natural abundance method to measure associative N_2-fixation depends on N_2-fixed by associative

microorganisms being predominantly taken up by the plant rather than going into the soil N pool (Shearer and Kohl, 1988). This point would also apply to the isotope dilution method.

Abbadie et al. (1992) used the natural abundance method to assess the sources of N that can meet the annual requirements (70 kg N ha^{-1} $year^{-1}$) in a savannah grassland at a site on the Ivory Coast, West Africa. They found that roughly 7% (5 kg ha^{-1} $year^{-1}$) was derived from the mineralization of soil organic matter and the same amount again from bulk precipitation. Approximately 17% (12 kg N ha^{-1} $year^{-1}$) was obtained via non-symbiotic N_2-fixation and the rest (roughly 40 kg N ha^{-1} $year^{-1}$) came from recycling of dead roots. In another field experiment using natural abundance, Russow et al. (2005) observed N_2-fixation rates in cyanobacteria-lichen crusts of the Negev Desert, Israel, ranging from 10 to 41 kg N ha^{-1} $year^{-1}$. Application of the ^{15}N natural abundance technique in oil palms in the field in Brazil identified diazotrophs with a high potential for N_2-fixation, but estimates of N_2-fixation could not be calculated because of the absence of suitable reference plant (de Carvalho et al., 2008), highlighting a significant limitation of this method.

2.2.5 N-fixed by difference method

In their critique of methods for measuring non-symbiotic N_2-fixation, Giller and Merckx (2003) suggested that the ultimate test of the contribution of N from fixation is to measure net inputs of N over long periods in the field (i.e., an N budget). However, there can be significant difficulties in controlling and measuring all the processes that contribute to N in the soil. A large contributor to error can be dependent on the volume of soil used (Dart, 1986). The total N balance technique does not measure directly the incorporation of fixed N into plant tissue or the soil. Any net increase is the sum of gains from various sources including fixation and unmeasured losses. Nonetheless, there are studies showing considerable N gains that can only be explained by inputs from N_2-fixation (e.g., Parker, 1957; Dart, 1986; Shabaev, 1986; Boddey, 1987, Boddey et al., 1995; Gupta et al., 2006).

There are very few published N-balance studies for cereal or grass production under field conditions. To achieve a reliable N balance it is necessary to monitor N inputs and outputs over a long term to account for variability and errors associated with small changes in soil N relative to the total (Vallis, 1973; Dart, 1986). It is also necessary to have a very high repeatability and

accuracy of N measurements through strict sampling protocols and extremely high sample numbers.

From a scientific viewpoint, N balance studies may not give a direct measure of N gains due to non-symbiotic N_2-fixation unless all inputs and outputs can be measured in detail over time. However, it does provide a net balance of N accumulation, which from a grower's perspective, is valuable information for planning N fertilizer inputs for a crop.

2.2.6 Use of combinations of techniques to substantiate measures of N_2-fixation

Used in conjunction with other measures such as the acetylene (C_2H_2) reduction assay, N budgets may increase the certainty of estimates. For example, Shearman et al. (1979) found that in grass pastures inoculated with a range of known N_2 fixing bacteria, rates of C_2H_2 reduction were strongly correlated (r=0.92) with nitrogen accumulation measured by the Kjeldahl method.

Nitrogen balance studies aided with ^{15}N have been used to strengthen evidence for associative BNF in sugar cane. Sugar cane plants were grown in soil in large pots, to which ^{15}N labeled fertilizer was applied (Lima et al., 1987; Urquiaga et al., 1992; Boddey et al., 1995). There was good agreement between estimates of biological N_2-fixation from N balance and isotope dilution (Urquiaga, 1992), but the authors were careful not to assume the same rates of fixation occurred in the field because the conditions of the experiment differed from those in the field (Lima et al., 1987). The use of $^{15}N_2$ gas in combination with C_2H_2 reduction assays to verify rates of N_2 fixed has been discussed earlier in this review.

Much of the information on estimates of N_2-fixation using techniques that are currently available apply to one instant in space and time. However, knowledge of the conditions that favor N_2-fixation and the rates at which fixation responds to changes in environmental conditions can be used to obtain estimates for a wider region if environmental conditions in those regions are known (such as meteorological records, cropping statistics and soil maps). Gupta et al. (2006) used this principle to derive estimates for parts of the southern agroecological zones of Australia. Using information from other studies on the effects of different soil moistures, temperatures and carbon sources, potential N_2-fixation in different zones was determined using a GIS spatial analytical tool. Use of this principle with a range of measurement strategies may

provide useful information about regions that are most likely to benefit from non-symbiotic N_2-fixation and where new advances can be made.

2.2.7 New technologies and approaches

Earlier in this review, research was cited where new molecular techniques have identified a whole suite of new non-culturable diazotrophic microorganisms (Bürgmann et al., 2004; Buckley et al., 2007; Roesch et al., 2008). These studies utilized *nifH* (which is a gene encoding enzymes involved in the fixation of atmospheric N; Bürgmann et al., 2004), specific polymerase chain reaction (PCR) and stable $^{15}N_2$ isotopic probing. The *nifH* gene is one of the structural genes for the evolutionarily conserved nitrogenase protein responsible for N_2-fixation (Zhang et al., 2007). The identification of a wide range of *nifH* containing microorganisms does not in itself tell us how much non-symbiotic N_2-fixation is occurring or possible. However, new techniques using microarray technologies have the potential to simultaneously measure the dynamics and/or activities of most microbial populations in the complex soil environment (Gentry et al., 2006; He et al., 2007). Zhang et al. (2007) used this approach to develop a *nifH*-based short oligonucleotide microarray as a rapid tool to monitor N_2-fixing diazotrophic populations in a wide range of environments. Their research showed that this technique will allow quantification and mapping of the abundance, diversity and activities of N_2-fixing populations. This approach is likely to assist in the identification of regions and managements that favor inputs of N from non-symbiotic N_2-fixation.

2.3 Factors affecting non-symbiotic N_2-fixation

2.3.1 Oxygen

Nitrogenase proteins are extremely sensitive to O_2 and on exposure to air the iron (Fe) protein is rapidly and irreversibly inactivated (Eady, 1980). Therefore, non-symbiotic N_2-fixing bacteria need mechanisms to exclude O_2 from within the cell before N_2-fixation (nitrogenase activity) can occur. There are just a few of the culturable bacteria that can fix N aerobically (*Azotobacter, Azomonas, Beijerinckia* and *Derxia*) (Stewart, 1980; Havelka et al., 1982). The first two genera have large cells that grow rapidly and exclude O_2 through rapid respiration and

the formation of some extracellular polysaccharide (Postgate, 1971; Dalton, 1980). The latter two genera produce copious amounts of slime and have large internal lipid bodies (Dalton, 1980). The remaining majority of culturable diazotrophic bacteria are much less competent at excluding O_2 and will only fix N under microaerobic or anaerobic conditions even if other growth processes occur aerobically (Dalton, 1980).

The need to exclude O_2 can place significant restrictions on when and where non-symbiotic N_2-fixation can occur. Anaerobic conditions can be created by saturating soil. Substantial amounts of N_2-fixation have been measured under these conditions (Rice and Paul, 1972). However, anaerobic conditions do not favor plant growth to facilitate O_2 transport. In drier soils much more subtle mechanisms are required.

2.3.2 Soil aggregates

Soils are not homogeneous. Particulate organic matter derived from plants and enriched labile organic fractions from soil microorganisms interact to form various classes of aggregates within the soil (Six et al., 2001). Macroaggregates are more common in undisturbed soils and C concentrations are more concentrated in macroaggregates than in microaggregates (Six et al., 2000). Aggregate dynamics and their relationship with microbial communities are central to the dynamics of soil organic matter (Denef et al., 2001). Aggregate formation in the soil is the means by which microaerobic and anaerobic conditions can coexist simultaneously with aerobic conditions in aerated soils. Limitations of gas diffusion in soil aggregates and root tissues result in low O_2 concentrations in sites of microbial consumption (Angert et al., 2001), resulting in steep O_2 gradients (Sierra and Renault, 1996; van der Lee et al., 1999). However, despite limitations of gas diffusion, substrates such as dissolved organic C can be allocated into both aerobic and anaerobic fractions and processes (Li et al., 2000). Therefore, it is possible for soluble products from organic matter decomposition, occurring under aerobic conditions, to supply C energy to microaerobic and anaerobic N_2-fixing bacteria within aggregates.

2.3.3 Minerals and other nutrients

It is well known that combined N in soil can inhibit N_2-fixation by non-symbiotic microorganisms (Knowles, 1980). However, the dynamics of N_2-fixing microbial populations are strongly linked to C:N ratios. Kavadia et al. (2007) showed that when C was abundant,

excess ammonium N was assimilated for growth needs by the microbial population and N_2-fixing microorganisms survived and even fixed N_2. However, under the same conditions but with low C, excess ammonium N concentrations inhibited the N_2-fixing population.

Products of decomposition of crop residues are often used as a source of energy for non-symbiotic N_2-fixation. Rates of decomposition can be slow, however, due to high C:N ratios (Roper and Ladha, 1995). Additions of mineral N to soils amended with crop residues with high C:N ratios increase the rate of decomposition (Sain and Broadbent, 1977; Barder and Crawford, 1981), making C available for use by N_2-fixing bacteria. Consequently, there is a fine balance between the supply of sufficient mineral N for proficient crop residue decomposition, without having a lot of excess mineral N that inhibits N_2-fixation.

Two other nutrients required for N_2-fixation are molybdenum (Mo) and Fe. They are both components of the nitrogenase enzyme, but they are rarely limiting in natural environments (Jensen, 1981). On the other hand, phosphorus (P) applications have been shown to increase significantly non-symbiotic N_2-fixation in soils (Smith, 1992; Reed et al., 2007a) and in grasslands (Eisele et al., 1989; Reed et al., 2007b) particularly in nutrient poor environments. Smith (1992) and Reed et al. (2007a) concluded that the strong inverse relationship between N_2-fixation and mineral N content in the soil is mitigated by the availability of P, and suggest that P is a key regulator of N biogeochemistry in soils.

2.3.4 Availability of C

Carbon is required as an energy source. Free-living N_2-fixing bacteria generally rely on decomposing plant material above and below ground, from crops and pastures. Associative N_2-fixing bacteria utilize root exudates within a rhizosphere association with plants and other organisms. Both environments are highly competitive with other microbial groups competing for limited energy resources. Endophytes on the other hand have access to nutrients from within the plant (Wilson, 1995).

Cellulose and hemicellulose are the major structural components of crop residues. A few species of N_2-fixing bacteria (*Azospirillum* spp.) are able to use straw directly for fixation (Halsall et al., 1985), but most N_2-fixing bacteria rely on decomposition to smaller components first. Organisms involved in decomposition of cellulolytic materials include a broad range of

bacteria, fungi, protozoa and microfauna. Almost all diazotrophic heterophic bacteria are able to utilize the products of cellulose decomposition, including carbohydrates and some organic acids and alcohols (Roper and Halsall, 1986). Rates of N_2-fixation are proportional to the amount of crop residue available and to rates of decomposition (Roper, 1983).

A key issue is whether sufficient C is available through the root system to sustain a meaningful level of N_2-fixation for crop growth. Estimates of the amount of C available and the efficiency of its use in N_2-fixation can be made, but the influence of factors such as competition between microorganisms for the limited amount of substrate available is difficult to predict (Dart, 1986). Estimates of efficiency are also difficult to make because they vary with the C substrate consumed, the O_2 tension and the physiological state of the bacteria.

Plants will also continuously secrete compounds into the rhizosphere in a process termed rhizodeposition. Root exudates include ions, free O_2 and water, enzymes, mucilage and a diverse array of C-containing metabolites (Bertin et al., 2003; Bais et al., 2006). Fixation is dependent on the proportion of N_2-fixing bacteria in the rhizosphere population. If this could be enhanced, more N becomes available.

2.3.5 Moisture

All microorganisms require moisture for growth and activity. High soil moistures have been used to promote N_2-fixation in soils by reducing O_2 at the sites of fixation (Rice and Paul, 1972). However, the importance of maintaining aggregate structure and O_2 gradients in unsaturated soils was highlighted by experiments assessing the moisture requirements for N_2-fixation in soils from the same site, both in field and in laboratory assays. In disturbed soils in the laboratory, a minimum of 50% field capacity moisture was required for nitrogenase activity (Roper, 1985), whereas in *in situ* assays in undisturbed soils in the field, nitrogenase activity occurred at moistures below 30% field capacity (Roper, 1983).

In some environments, N_2-fixing bacteria have adapted to harsh semi-arid environments where lichens (containing cyanobacteria or other free-living bacteria in association with a fungus) can contribute significant amounts of biologically fixed N (Rychert et al., 1978; Russow et al., 2005). Another significant adaptation to dry environments is rhizosheaths around the roots of several species of perennial grasses. Rhizosheaths are composed of sand grains cemented

around the root through bonding agents such as mucilage and other root exudates. Compared to the surrounding sand, rhizosheaths support enriched organic materials, greater moisture contents and a higher density of micro-organisms including associative diazotrophs (Othman et al., 2004). Amounts of N_2-fixed were small, but because of the isolation of this activity within the rhizosheath away from the bulk soil, the grass plants are likely to be the primary beneficiaries of the N_2-fixed.

2.3.6 Temperature

Nitrogen fixation has been shown to occur in extreme temperatures from near 0°C in Antarctica to desert environments. In deserts, most N_2-fixation occurs in the cooler hours of the morning, utilizing dew or after summer rains (Rychert et al., 1978), but N_2-fixing microorganisms survive during the intervening hot dry conditions up to 60°C (Jensen, 1981).

Nitrogen fixation responded significantly to temperature (Roper, 1983). Laboratory experiments indicated that the most favorable temperatures for N_2-fixation were between 30 and 35°C, with a range from 4 to 45°C (Roper, 1985), although there seemed to be a shift in temperature ranges for activity consistent with climatic temperature ranges at each of the sites from which samples were collected. Temperature ranges for activity vary in different studies around the world but the main consensus is that activity is best between 25 and 37°C (Jensen, 1981).

2.3.7 Management Practices

No-tillage supports the stability of macro-aggregates, but any increase in soil disturbance reduces aggregation, reduces soil C, and disrupts the soil pore network by which soil organisms interact (Six et al., 2000; 2001; Young and Ritz, 2000). Soil water contents are conserved under no-till compared with cultivated soil. As a result of all of these factors, biological activity under no-till is characteristically higher than in cultivated soils (Young and Ritz, 2000). However, biological changes in response to altered tillage practices can be slow, sometimes taking several years to develop (Cookson et al., 2008).

Roper et al. (1989) evaluated N_2-fixation by free-living bacteria using crop residues as an energy source, in field trials under different tillage practices [stubble burnt and cultivated,

stubble incorporated (disc ploughing), scarification (light mixing of soil and straw at the surface) and no-tillage]. One year after imposition of the tillage treatments for the first time, nitrogenase activity was highest in the incorporated treatment where there was good soil-straw contact and hence good microbe-straw contact. But five years later, N_2-fixation was favored under the less disturbed scarified treatment. Activity under no-tillage was slightly less than the scarified treatment but activity in the 'incorporated' treatment significantly lower. This suggests that there were changes in soil structure and microbial function over time within each tillage treatment. One can hypothesize that in the future, had the experiment continued, the no-tillage treatment may function best. This certainly was the case in a field experiment conducted by Lamb et al. (1987) at a site where tillage (no-till, stubble mulch and plough) had been established twelve years prior to the experiment. The amount of N_2-fixed was significantly greater under no-till compared with the disturbed soils.

In no-till systems there can be inputs of N other than from the soil or rhizosphere. Populations of soil macro fauna such as ants and earthworms have been consistently shown to be more abundant in no-till soils (Francis and Knight, 1993; Fraser, 1994; Young and Ritz, 2000). Some of these organisms live on diets with an extremely high C:N ratio and must obtain N from some other source. Measurements using *nifH* homologs, total N and C_2H_2 reduction assays suggest significant amounts of N_2-fixation occur in the guts of earthworms (Striganova et al., 1993).

In a review, Ball et al. (2005) indicated that crop rotation profoundly modifies the soil environment by influencing the removal of nutrients from the soil, return of crop residues (including quality), development and distribution of biopores, and dynamics of microbial communities. Therefore, it is likely that crop rotations will affect the potential for N_2-fixation. However, Ball et al. (2005) acknowledge that further research is needed to fully understand the impact of crop sequences on the fate of organic N in soil.

2.3.8 Plant Genotypes

It has been suggested that associative N_2-fixation may be under the genetic control of the host plant (Chaulk, 1991; Belimov et al., 1995). Differences in associative N_2-fixation have been observed between different lines of rice (Baldani and Baldani, 2005; Knauth et al., 2005), wheat

(Neal and Larson, 1976), maize and sorghum (Krotzky et al., 1986; Werner et al., 1989) and weeds (Conklin and Biswas, 1978). Sukiman and New (1990) found that in addition to differences in efficiency of adsorption between bacterial strains, there were differences between different host plants, and even between different parts of the roots of a single host plant, when bacterial strains were ranked in order of numbers adsorbed. This confirms and extends previous findings that adsorption of *Azospirilla* is affected by bacterial strain, host plant, and part of the root involved (Umali-Garcia et al., 1980; Jain and Patriquin, 1984; Bashan and Levanony, 1989), using different host plants and bacterial strains.

Characteristics which contribute to high N_2-fixing genotypes were a reduced transpiration rate, lower number of stomata, and increased root exudates with a high concentration of dicarboxylic acids (Krotzky et al., 1988; Werner et al., 1989). Wood et al. (2001) suggested that plants with an increased release of photosynthate to their rhizosphere should be a priority for the future development of broad-acre agricultural systems that are more self-sufficient for N nutrition.

2.4 Transfer of fixed N from diazotrophs to plant and other organisms

The transfer to plants of N_2-fixed non-symbiotically is likely to depend very much on the location at which N_2-fixation occurs. Endophytic diazotrophs are likely to be able to supply biologically fixed N directly to the host (Sturz et al., 2000). However, James (2000) indicated that there is no evidence for a direct transfer of N from endophytic diazotrophs as in a *Rhizobium*-legume symbiosis. Endophytic diazotrophs have only been observed in intercellular spaces, vascular tissue, aerenchyma and dead cells, and not within living host cells (McCully, 2001). As such, N transfer from these organisms is likely to be dependent on the death and release of fixed N to the plant (James, 2000). The only exception to this is ammonium-excreting mutants of diazotrophs, for example endophytic *Azospirillum* in para-nodulated cereals (Sriskandarojah et al., 1993; Christiansen-Weniger, 1998), but it is unlikely that these organisms could survive and fix N under the field conditions if used as inoculants. Further integrated multidisciplinary research is needed to assess the inputs of N from endophytic diazotrophs (James, 2000).

Transfer to plants of N_2-fixed by diazotrophs, or N contained in non-fixing microbial biomass in the soil or rhizosphere, is usually dependant on the death of these bacteria and release of ammonium or amino acids. Significant quantities of fixed N can be released this way (Lethbridge and Davidson, 1983a; 1983b). However there are some examples of diazotrophic bacteria which are capable of excreting nitrogenous substances during growth, for example *Berjerinckia derixii* (Miyasaka et al., 2003) and cyanobacteria (Jones and Wilson, 1978; Balachandar et al., 2004).

In rhizosphere associations, N_2-fixed can either be directly taken up by the plant or remain in the surrounding soil N pool. There is little information about the proportions of N transfer to each of these pools. However, transfer of fixed N to plants from associative N_2-fixing bacteria has been demonstrated using $^{15}N_2$ by Giller et al. (1984; 1988) and others reviewed by Boddey (1987) and James (2000). Release of N following the death of diazotrophic bacteria in the rhizosphere can be rapid due to wetting and drying cycles and microbial competition.

2.5 Enhancing the value of non-symbiotic N_2-fixation

2.5.1 Inoculation

Diazotroph inoculant is a formulation containing one or more bacterial strains or species, in an easy-to-use and economical carrier material, either an organic material or a synthesis of defined molecules. The inoculant is the means of bacterial transport from the factory/laboratory to the living plant. Inoculant formulation has a crucial effect on the inoculation process because the chosen formulation determines potential success of the inoculant (Bashan, 1998).

Root colonization of beneficial microbes is always considered a major factor in successful inoculation of plants by bacteria (Suslow, 1982). For *Azospirillum* the inoculation site is likely to determine its ultimate fate: colonizing the roots and surviving, or dying. During this establishment period, the bacteria are exposed to the natural physical forces and interactions that prevail between soil bacteria and soil particles, like adsorption, encapsulation by clay minerals, and wet and dry regimes of the soil.

Bacterial movement can play a significant role in the survivability of bacteria in the soil and rhizosphere, where attachment is also very important for beneficial effects (Turnbull et al., 2001). The majority of the PGPR-non-legume association bacteria form colonies around the root superficially and in a few cases extend internally (Gallo and Fendrik, 1994; Mia et al., 1999). The close association between a plant and an endophyte may provide suitable conditions for nutrient transfer between the bacteria and their host, than the association between predominantly rhizosphere bacteria and plants (Stoltzfus and de Bruijn, 2000).

There have been many studies on inoculation with N_2-fixing bacteria of non-legumes (predominantly cereals and grasses), with reported above-ground increases in total plant dry weight, plant N in shoot and grain, number of tillers, ears, spikes and grains per spike, and increases in root length and volume (Bashan and Levanony, 1990). The most successful inoculation responses have been in plot trials under controlled conditions (e.g., Negi et al., 1987; Aly et al., 1999; Alam et al., 2001; Yanni et al., 2001). Inoculation experiments in the field have been less consistent. For example, Baldani et al. (1987) found increases in grain yield in wheat of up to 31% following inoculation with *Azospirillum* spp., but due to variability in the trials, there were no statistical differences between inoculated plants and untreated controls. Das and Saha (2003) observed increases of up to 20% in grain yield in rice in response to inoculation with *Azotobacter* spp. and *Azospirillum* spp., but these increases were less than those recorded with optimum N fertilizer application. Andrews et al. (2003) concluded that currently no non-symbiotic N_2-fixing bacterial inoculant is available that can match the consistency of N fertilizers for reducing soil N deficiency.

A key factor to the success of inoculation with *Azospirillum* is the choice of bacterial strain(s). Even though no specificity between plant species and bacterial strains has been demonstrated, some affinity exists between bacteria and plant species (Penot et al., 1992) or even cultivars (Wani et al., 1985). Effects of plant genotype on the interaction with *Azospirillum* have been demonstrated, for example, for wheat (Kapulnik et al., 1987; Caballero-Mellado et al., 1992), maize (de Salamone et al., 1996), and pearl millet (Bouton et al., 1985).

One of the difficulties of inoculating soils with bacteria is that the inoculants generally decline rapidly due to competition with the native microflora (Schank and Smith, 1984; Rao et al., 1987). Inoculants compete for available nutrients or become food for indigenous micro- and

macro-fauna. Hence the ultimate test for even the most effective beneficial organism is the ability to survive and colonize plant roots in the presence of much larger populations of indigenous microorganisms (Bashan and Levanony, 1990). The delivery of inoculants with organic matter, either as a compost or peat, has been shown to improve the performance of some inoculants (Hadar, 1986; Negi et al., 1987). Inoculum formulation and application technology are likely to be crucial for inoculant survival and success.

2.5.2 Endophytes and genetically modified organisms

There have been a lot of advances in the physiology, biochemistry and genetics mostly associated with *Azospirillum* species in the last decade. In addition, the role of these bacteria in the interaction with gramineae plants and other non-legume plants has been confirmed (Vande Broek and Vanderleyden, 1995; Bashan and Holguin, 1997a; Steenhoudt and Vanderleyden, 2000).

As stated earlier in this review, microorganisms that associate with a host plant in an endophytic relationship are more likely to be successful inoculants because they can escape competition from indigenous microflora. Increased success with endophytic inoculants may be possible through genetic manipulation. Bloemberg (2007) studied plant growth promoting bacteria, including N_2-fixing bacteria in their natural environment. Mia and Shamsuddin (2009) have manipulated N_2-fixing genes into rice systems with a rice-*Rhizobia* symbiosis system. The recent advances in accepting symbiosis at the molecular level, and the ability to manipulate genes in the rice for fixing N, have created tremendous opportunities. This association lead to greater production of vegetative and reproductive biomass due to rhizobial modulation of the plant root architecture, enhanced root hairs for absorbing plant nutrients efficiently rather than BNF per se (Sheng and Huang, 2001; Yanni et al., 2001; Mia and Shamsuddin, 2009).

Other advances using molecular strategies may be possible. For example, the creation of ammonium excreting mutants of diazotrophs in which the mechanisms, by which ammonium inhibits N_2-fixation, are disarmed (Colnaghi et al., 1997) or the increased production of nitrogenase reductase such as in an *Azospirillum brasilense* mutant (de Campos et al., 2006). The transfer of *nif* genes, along with others for functional N_2-fixation, was considered to be the most suitable strategy to achieve symbiotic N_2-fixation in non-legumes (Saikia and Jain, 2007).

However, the highly complicated system of gene regulation, and the fate and survival of such mutants in the field is uncertain (Jaimes, 2000). Genetic engineering through biotechnological means has seen little or no success in achieving the induction of symbiosis between cereals and diazotrophs.

2.5.3 Co-inoculation

Even greater benefits may be possible where inoculants have a dual benefit, through increased N nutrition via N_2-fixation coupled with the production of plant growth hormones. There are several groups of organisms that are known to fix N, but also produce phytohormones and /or provide protection against fungal and bacterial pathogens (e.g., Dobbelaere et al., 2003; Vessey, 2003; Ahmad et al., 2006; de Campos et al., 2006; Bloemberg, 2007; Buckley et al., 2007).

Azospirillum has been mixed with other microorganisms which have a proven effect on plants, such as *Rhizobium* and mycorrhizal fungi, in order to enhance the effectiveness of the latter (Barea et al., 1983; Plazinski and Rolfe, 1985; del Gallo and Fabri, 1991). This role of "helper" bacteria is especially suitable for *Azospirillum* since its primary effect is on increased root development (higher surface area, more root hairs, and increased excretion of root exudates), thus increasing the probability of successful infection by the major contributor in a synergistic way. Inoculation consortia apparently work better when P-solubilizing bacteria, *Azobacter*, rhizobia, bacilli, and VAM fungi are incorporated, perhaps aiding in the growth of each other by synergistically providing nutrients, removing inhibitory products, and in the process, enhancing plants' ability to grow better (Muthukumarasamy et al., 2002; Bashan et al., 2004). Data is still insufficient in this area to justify full-scale field experiments with reasonable chances of success (Bashan, 1993).

2.5.4 Unculturable microorganisms

Microbes are not always culturable even though their biological activities may be detectable (Pace, 1997; Barer and Harwood, 1999). Earlier it was pointed out that the most effective N_2-fixing microorganisms, identified using new molecular technologies, are predominantly unculturable bacteria (Hamelin et al., 2002; Hurek et al., 2002; Knauth et al., 2005; Buckley et al., 2007). If this is confirmed by future studies, the development of effective

inoculants becomes difficult unless some means of transferring these organisms from one plant to another can be devised. Advances in culturing technology (e.g., Janssen, 2006) offer a new hope. Also new molecular technologies may enable the transfer of effective N_2-fixing characteristics from unculturable to culturable organisms. However, there are other approaches which may enhance inputs of N via non-symbiotic N_2-fixation by those unculturable organisms.

2.5.5 Plant breeding

Only in Brazil are there varieties of sugar cane that have been shown to fix over 60% of their N, which is roughly over 150 kg N ha^{-1} $year^{-1}$ (Boddey et al., 1995). Elsewhere in the world, measurements of contributions to N supply in sugar cane via biological N_2-fixation have been small (Biggs et al., 2000), although specific associations between diazotrophic bacteria and sugar cane have been observed (Li and Macrae, 1991). It has been argued that this may be due to sugar crops in Brazil being systematically bred for higher yields with low fertilizer inputs (Boddey et al., 1995; Baldani et al., 2002). Baladani et al. (2002) suggest that such breeding processes with low fertilizer inputs has led to the development of (or preserved) an effective association between N_2-fixing bacteria and the plant.

Almost all of our modern crop varieties have been developed in conjunction with the use of fertilizers. Has the capacity for significant levels of associative N_2-fixation been lost through breeding processes? Certainly there are other mechanisms that have been shown to be lost this way. For example, *Leymous racemosus*, a wild relative of wheat, barley and rye, is able to produce biological nitrification inhibitors which have been shown to protect N in soil from losses via nitrous oxide release and leaching (Subbarao et al., 2007). However, this ability has been lost in our modern cereal lines. Efforts are now focused on reintroducing these traits into cultivated wheat varieties (Fillery, 2007; Subbarao et al., 2007). Should we be examining the capacity for associative and endophytic N_2-fixation in some of the wild relatives of wheat and other cereals?

Further support for this notion has been found in rice. Knauth et al. (2005) examined the composition of diazotrophic communities associated with related rice cultivars (*Oryza sativa*) and wild species (*Oryza brachyantha*), and found that when grown under identical conditions in the same soil, without N fertilizer, there were remarkable differences in root associated *nif*H

fragments expressed in the wild species of rice roots. This indicated that the active diazotrophs were not related to cultured strains. Anyia et al. (2004) studied the responsiveness of Canadian cultivars of wheat to inoculation with *Azorhizobium caulinodans*. The results showed considerable growth promotion by *A. caulinodans* on wheat cultivar CDC Teal. The wheat variety AC Taber, on the other hand, did not benefit from inoculation, which suggests that growth promotion may be cultivar specific.

Evidence that ecosystems with low N promote non-symbiotic N_2-fixation occurs in a perennial grass (*Molinia coerulea*), which grows in extremely oligotrophic environments. Hamlin et al. (2002) observed that the rhizosphere of these grasses supported a diversity of N_2 fixing bacteria, 56% of which contained *nif*H sequences that did not match any cultivated diazotrophs, but were dominant in the roots and surrounding soil. Further examination of such oligotrophic systems may yield material that can be used to breed plants that support substantial levels of associative N_2-fixation.

2.5.6 Conserving fixed N (reducing N losses)

The value of non-symbiotic N_2-fixation, even if the rates are small, will be greatly enhanced if N losses from soil are reduced or eliminated. There are numerous publications describing substantial losses of N that occur via denitrification and loss to the atmosphere as NO and N_2O or by leaching of mobile NO^-_3 ions below the root zone. Some mechanisms to reduce N losses from the soil are known, others need to be developed.

Retention of crop residues with high C:N ratios not only support non-symbiotic N_2-fixation, but can immobilize soil N for release and use by new crops later on. Early research by Allison and Klein (1962) demonstrated that following the addition of wheat straw to soil, N was within the first 7 days immobilized by growing populations of decomposer microorganisms. Nitrogen release commenced about 25 days later but was gradual, over 6 to 8 weeks, a process that is more likely to benefit a new crop and avoid N losses than a single large input of N.

2.6 Conclusion

There is much literature on the subject of non-symbiotic N_2-fixation, and a wide range of inputs from this source are claimed (van Berkum and Bohlool, 1980; Bashan, 1998; Belnap, 2001). A historical analysis of studies on non-symbiotic N_2-fixation in Graminaceous plants demonstrates significant advances in several aspects of plant/bacteria interactions. However, the expectation that nitrogen fixation efficiency might be equivalent to rhizobia/legume symbiosis did not turn out to be true, although the endophytic diazotrophic bacteria/plant association show some characteristics that are similar to the legume symbiosis.

Environmental and management factors play an enormous role in microbial function, but it is likely that differences in methodology, including application of individual methods, have contributed to some of this variability. This review has attempted to highlight the values of existing methodologies and their proper use. In particular, combinations of methodologies have been most useful in determining absolute amounts of N_2-fixed and verifying other measures such as the C_2H_2 reductase assay and N balances. Expensive ^{15}N methodologies are not feasible on a large scale, but their use in combination with affordable and sensitive assays, such as C_2H_2 reduction assay, is critical to building our knowledge of non-symbiotic N_2-fixation further. Verification of the C_2H_2 reduction assay using $^{15}N_2$ should be done as a matter of course to determine conversion ratios of C_2H_4:N_2, although there are still some difficulties in achieving this for free-living N_2-fixing bacteria in the soil. Nonetheless, research shows that C_2H_4:N_2 ratios are generally 1 to 2 times the theoretical range of 3 to 4, except in anaerobic environments when conversion factors are much larger, and therefore can result in considerable overestimates of N_2-fixation. New technologies using molecular approaches, particularly when combined with current methods, will broaden further our understanding of non-symbiotic N_2-fixation. In particular, microarray technologies offer the opportunity to investigate simultaneously the diversity and function of diazotrophic microbial communities.

Generally there is a good understanding of the environmental factors controlling non-symbiotic N_2-fixation, and this can be helpful in designing farming systems that promote N inputs from fixation. Farming practices have changed over the last twenty years towards intensive cropping, no-tillage and stubble retention. These changed practices provide increased C inputs, a key driver for non-symbiotic N_2-fixation. Reduced N mineralization over summer,

associated with intensive non-legume rotations, and increased available P, are further drivers of N_2-fixation. A lack of soil disturbance associated with no-tillage practices promotes a well-developed soil structure with large aggregates, which enable aerobic cellulytic functions and anaerobic/microaerobic N_2-fixation to occur in close proximity to each other. These practices also conserve soil moisture and maintain microbial activities and interactions. It is important to re-evaluate non-symbiotic N_2-fixation under these changed farming practices, and to evaluate alternative systems, such as pasture cropping, which may require low N fertilizer inputs due to gains from N_2-fixation but also reductions in N losses.

Further gains may be possible through inoculation with highly efficient N_2-fixing bacteria, particularly if they have the additional capacity to promote plant growth through the production of phytohormones. However, the ultimate test for even the most beneficial inoculants is to be able to survive in soil and colonize plant roots. Inoculation with bacteria that can form an endophytic relationship within the plant may increase the potential for success. However, many effective diazotrophic bacteria remain unculturable and this may limit our ability to exploit them as inoculants unless new culturing techniques can be developed, or alternative means of transferring them to other plants can be found.

There is evidence that some early wild relatives of some of our modern cereal varieties, or plants that have been bred with low N fertilizer inputs, have a greater capacity to fix N symbiotically than those varieties that have been bred in conjunction with the use of fertilizers. Further, it has been observed that these wild types support significant populations of unculturable diazotrophic bacteria which appear to be superior to culturable diazotrophs. Re-introduction into modern varieties of traits that promote the colonization of highly efficient, but unculturable diazotrophic populations may be achievable through plant breeding.

Any strategies to increase non-symbiotic N_2-fixation should be coupled with mechanisms that reduce N losses from the soil, so that maximum possible benefits from non-symbiotic N_2-fixation might be achieved. Defining the functionality of genes present in nitrogen fixing bacteria, as well as knowledge generated by genome sequences of several plants of agronomic interest, should contribute to a better understanding of these associations, particularly the endophytic ones.

3. COMPATIBILITY OF TWO DIAZOTROPHS, *GLUCONACETOBACTER AZOTOCAPTANS* AND *AZOSPIRILLUM LIPOFERUM*, WITH THE SEED APPLIED TREATMENT DIVIDEND® XL RTA®

3.1 Preface

The objective of this study was to quantify survival of *Gluconacetobacter azotocaptans* and *Azospirillum lipoferum* inoculated onto sterilized and unsterilized wheat seed (*Triticum aestivum* cv Lillian), as well as on wheat that was treated with the fungicide Dividend XL® RTA®. Based on recovery of colony forming units (CFU), it could be determined if *G. azotocaptans* or *A. lipoferum* were compatible with Dividend XL® RTA®, and if they were able to compete against naturally occurring microorganisms on the seed coat of wheat. Survivability of *G. azotocaptans* and *A. lipoferum* was also determined by recovering CFU from wheat seeds over a length of time after inoculation.

3.2 Introduction

Microbial inoculants can be used in agriculture to enhance the availability of macronutrients (Adesemoye and Kloepper, 2009; Khan et al., 2009; Mahdi et al., 2010), or to promote plant growth by stimulating plant hormone production (Bashan and Holguin, 1997a; Sullivan, 2001; Ortiz-Castro et al., 2009; Hayat et al., 2010; Saharan and Nehra, 2011). Diazotrophs are bacteria or archaea that fix atmospheric nitrogen (N_2) to plant available forms (Postgate, 1998). They are free-living plant-growth-promoting-bacteria (PGPB) capable of influencing growth and yield of numerous plant species including rice (*Oryza sativa*), sugar cane (*Saccharum officinarum*), corn (*Zea mays*), and coffee (*Coffea arabica*) (Cavalcante and Dobereiner, 1988; Fuentes-Ramires et al., 2001; Mehnaz and Lazarovits, 2006). *Azospirillum lipoferum* are routinely isolated from agricultural lands and crop plants including cereals (Nath et al., 1997; Weber et al., 1999). *Gluconacetobacter azotocaptans* colonize the root cells of sugar cane (James et al., 2000; Anitha and Thangaraju, 2010). Sugar cane belongs to the grass family Poaceae like rice, corn and wheat which leads to speculation that these other grasses might also respond to *G. azotocaptans* (Arencibia et al., 2006).

There are mixtures of factors that affect N_2-fixation by PGPB. One of those factors is pesticides including herbicides, insecticides, fungicides, and biocides. Among agricultural practices, the widespread use of plant protection products represents a potential threat to soil organisms (Chalam et al., 1997; Hashem et al., 1997; Gomes et al., 1998; Lors et al., 2005). Many fungicides are applied directly to seed to reduce the establishment of seed- and soil-borne disease organisms (Ahmad and Khan, 2010). However, active ingredients in fungicides as well as their formulation materials (i.e., colorants) can be toxic to microorganisms applied as inoculants, which in turn can inhibit soil N_2-fixation (Wani et al., 2005).

The definition of compatibility is the ability to exist together in harmony (Patterson and Litt, 1989). Compatibility between a seed-applied fungicide and a microorganism is the ability to coexist together around the seed without any toxic effects from the pesticide on the microorganisms. Compatibility can be influenced by the length of time the microorganism is in direct contact with the seed (Curley and Burton, 1975).

Dividend® XL RTA® (Syngenta Crop Protection Canada, Guelph, ON), with active ingredients difenoconazole at 15 mg L^{-1} and mefenoxam at 26 g L^{-1}, is a dual-purpose seed treatment, frequently used on wheat to protect against infection by the soil pathogens

Cochliobolus spp., *Fusarium* and *Pythium* (Saskatchewan Agriculture, Food and Rural Revitalization, 2010). Damage from these diseases can reduce plant quality and cause yield losses (van der Burgt and Timmermans, 2009). To date there have been no compatibility tests published with Dividend® XL RTA® and *G. azotocaptans* or *A. lipoferum*. Survival of the diazotroph strains with prolonged contact with the fungicide was examined to determine if there was compatibility, or if the fungicide negatively impacted the diazotroph strains.

Sterilized seed contains no microorganisms on the seed coat. Therefore inoculation with a diazotroph strain would allow for survival of *G. azotocaptans* or *A. lipoferum* without competition or predation from other microorganisms. A comparison between a fungicide seed treatment and diazotroph strains versus only diazotroph strains on sterilized seed was done to illustrate if there was some interaction of the fungicide and inoculant on seed. However, sterilizing seed is not a practice used by producers; thus, application of the diazotroph strains to non-sterilized seed shows basic interactions with other microorganisms that may be on the seed coat. A comparison between fungicide treated seed and diazotroph species survival versus survival of the diazotroph species under competition from naturally occurring seed coat microorganisms was conducted.

3.3 Materials and methods

3.3.1. Diazotroph strains

Azospirillum lipoferum and *G. azotocaptans* were isolated from the rhizosphere of corn growing in eastern Canada (Mehnaz and Lazarovits, 2006). Stock solutions of the organisms were made and stored at -80°C. Two 2 mL cryovials, one with *A. lipoferum* and one with *G. azotocaptans*, were removed from the freezer and thawed at room temperature. Liquid G5-4 growth media was prepared by mixing 2 L reverse osmosis (RO) water; 2 g mannitol; 1 g K_2HPO_4; 0.4 g $MgSO_4 \bullet 7H_20$; 0.2 g NaCl; 4 g yeast extract; 2 g glucose; 1 g arabinose; 0.14 g FeEDTA; 16 g glycerol; and 10 g gum Arabic in a 4 L beaker. One litre of solution was poured into a 2 L beaker. This solution was adjusted with NaOH to pH 7. The remaining 1 L of solution was poured into another beaker where NaOH was added to adjust to pH 6. These media were then autoclaved.

A 200 µL of thawed stock solution *G. azotocaptans* was pipetted into the pH 6 medium and shaken for 48 h at a speed of 200 rpm at 23±1°C. Also, 200 µL of the thawed stock solution

A. lipoferum was added to the pH 7 medium. It was shaken for 30 h at a speed of 200 rpm at 23±1°C. Solutions were stored at 15°C and plated onto solid C1 media for *G. azotocaptans*, and solid RC media for *A. lipoferum* to determine titres and to verify that the solutions were not contaminated before using in the experiments.

The C1 media contained 2 g L^{-1} glucose and yeast extract; 1.5 g L^{-1} glutamic acid and peptone, 0.5 g L^{-1} K_2HPO_4 and $MgSO_4 \cdot 7H_2O$; and 15 g L^{-1} agar. The RC media consisted of 5 g L^{-1} D-L malic acid; 4.8 g L^{-1} KOH; 0.5 g L^{-1} K_2HPO_4 and yeast extract; 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$; 0.1 g L^{-1} NaCl; 0.015 g L^{-1} $FeCl_3 \cdot 6H_2O$; 15 g L^{-1} agar; and 10 mL Congo red stock. The Congo red stock was made by adding 1 g Congo red to 100 mL RO water, sterilized in an autoclave, and added to the molten media. Based on earlier experiments conducted at Novozymes BioAg, media and incubation times were selected based on highest yield (CFU mL^{-1}).

3.3.2 Seed treatments

Wheat seed (*Triticum aestivum* cv. Lillian) was used in the experiment. Three main treatments were tested: i) sterilized seed; ii) non-sterilized seed with Dividend® XL RTA®; iii) non-sterilized seed with no fungicide. In separate experiments, *G. azotocaptans* or *A. lipoferum* were applied to each of the three seed treatments.

Wheat seed was sterilized by washing with 75% ethanol for 1 min, followed by a solution of 25% of 6% sodium hypochlorite and 75% water for 5 min. The solution was drained, and the seed washed 5 times with sterilized water. Seeds were placed in a sterilized, laminar flow hood until dry. Seed was weighed into 3 x 150 g batches each for sterilized seed, non-sterilized seed, and non-sterilized seed treated with Dividend® XL RTA® and placed in plastic Ziploc bags.

The fungicide seed treatment Dividend® XL RTA® was applied to non-sterilized seed at the manufacturer's recommended rate of 3.25 mL kg^{-1} of seed. The Dividend® XL RTA® was applied to the seed and mixed in a plastic Ziploc bag using a tumbling motion until all the seed was visibly, evenly coated. The fungicide was allowed to air dry on the seed by opening the bag for 5 min under a laminar flow hood.

Gluconacetobacter azotocaptans or *A. lipoferum* were pipetted from their liquid growth cultures onto non-sterilized, sterile and Dividend® XL RTA® seed treatments at a rate of 1 µL g^{-1}, with the aim of applying between 10^5 and 10^6 CFU $seed^{-1}$. The bags were then sealed and shaken vigorously using a tumbling motion to evenly cover the seed. The diazotroph treatments

were allowed to air dry for 5 min by opening the Ziploc bag under a laminar flow hood. Treated seed was stored in the bags at room temperature (21°C±2) during the experiment time period.

3.3.3 Survival of *Gluconacetobacter azotocaptans* and *Azospirillum lipoferum*

Standard plate count methods (Shin et al., 2007) were used to enumerate the CFU of *G. azotocaptans* and *A. lipoferum* at 0, 2, 4, 6, 24, 48, and 72 h after inoculation. A 10 g sample of wheat seed from the *G. azotocaptans* or *A. lipoferum* seed treatments was placed in a test tube containing 100 mL of 0.01% (vol/vol) Tween 80® solution (Fischer Scientific, Fair Lawn, NJ, USA). The mixture of seed and Tween 80® was sonicated for 2 min in an ultrasonic water bath and subsequently mixed with the aid of a shaker for 2 min to remove the inoculants from the seed. One millilitre of the Tween 80® containing *G. azotocaptans* or *A. lipoferum* from the wheat seed was added to 9 mL Tween 80® solution to make a 10^{-2} dilution. A 1 mL sample from the 10^{-2} dilution was added to 9 mL Tween 80® solution and vortexed to make a 10^{-3} dilution. A 1 mL sample from the 10^{-3} dilution was added to 9 mL Tween 80® solution and vortexed to make a 10^{-4} dilution. Between each dilution the samples were vortexed for 10 seconds. One millilitre dilution of 10^{-2}, 10^{-3}, and 10^{-4} were then plated on C1 or RC media. Four subsamples per dilution per treatment were plated for each time period. Plates were incubated for 4 d in a dark incubation chamber at 28±2°C. Colony forming units were recorded then converted to CFU g^{-1} seed.

3.3.4 Statistical analysis

Slopes (log CFU mL^{-1} versus time) of the linear portion of survival for each of the diazotroph species on the three seed treatments were compared by regression analysis. The data was checked for normality, based on the residuals, for goodness of fit as well as by using Shapiro-Wilk test. The statistics were tested at 95% confidence (P<0.05).

For each diazotroph species recovered from each seed treatment, a slope comparison was done. The formula used for this statistical test was from the work of Clogg et al. (1995),

$$Z = \frac{b_1 - b_2}{\sqrt{SEb_1^2 + SEb_2^2}} \qquad [3.1]$$

where b_1 is the estimated slope of one coefficient, b_2 is the estimated slope of a second coefficient, and the denominator is the square root of the addition of the standard error of b_1 squared and b_2 squared.

3.4 Results

For statistical reasons, it was decided that CFU mL^{-1} estimations were counted on spread plates only between 15 and 250 colonies (Mary Leggett, personal conversation). Colony formation at 0 to 24 h after inoculation was easily distinguishable for both *G. azotocaptans* and *A. lipoferum.* However, at 48 and 72 h after inoculation, there was some contamination on some of the plates which reduced the replicate numbers at each dilution.

The addition of *G. azotocaptans* to wheat treated with each of the three seed treatments resulted in different numbers of the organisms removed from the wheat seed immediately after application (i.e., time zero; Table 3.1). At time zero, it was found that there were $10^{5.17}$ CFU of the *G. azotocaptans* recovered from the sterile seed, equivalent to 86% recovery; $10^{5.65}$ CFU were obtained from non-sterilized seed, equivalent to 94% recovery; and $10^{5.08}$ CFU were recovered off of the Dividend® XL RTA® treated seed, equivalent to 84% recovery. Significant differences (P<0.05) were not observed between the amounts of *G. azotocaptans* CFU recovered off of the seed treatments and what was initially applied to the seed. Also 6 h after inoculation, there were no significant differences (P<0.05) observed in *G. azotocaptans* CFU recovery from the three wheat seed treatments. Nonetheless, there was significance (P<0.05) in the decline in CFU when comparing initial application rates and the recovery of CFU at 6 h after inoculation.

After time zero there was a steady decline in the retrieval of CFU off wheat seed with each seed treatment at each time point after the initial inoculation time point (Figure 3.1). Non-sterilized seed had the highest recovery of *G. azotocaptans* from 0 to 24 h after inoculation ($10^{5.65}$, $10^{5.60}$, $10^{5.19}$, $10^{4.57}$ and $10^{4.07}$ CFU recovered respectively). Therefore it appears that *G. azotocaptans* is able to survive among the naturally occurring microorganisms on the seed coat of the wheat seeds. Dividend® XL RTA® treated seed had the lowest recovery at each time point.

A slope comparison was done using equation 3.1. Results showed that there was no significant (P<0.05) difference in slopes between the three seed treatments for survival of *G.*

Table 3.1. Comparison of the initial application and recovery of *G. azotocaptans* (CFU mL^{-1}) from three wheat seed treatments at 0 and 6 hours after inoculation. The percentage of recovery uses the initial application of 10^6 CFU mL^{-1} as 100%.

Treatment	Recovery at 0 h		Recovery at 6 h	
	Log CFU mL^{-1}	%	Log CFU mL^{-1}	%
Initial application on all treatments	6.00a †			
Sterile Seed	5.17a	86	4.01b‡	67
Dividend® XL RTA®	5.08a	84	4.07b	68
Non-sterilized Seed	5.65a	94	4.57b	76

†Means followed by the same letter are not significantly different ($P<0.05$) at time zero.
‡Means followed by the same letter are not significantly different ($P<0.05$) at 6 h after inoculation compared to the initial application.

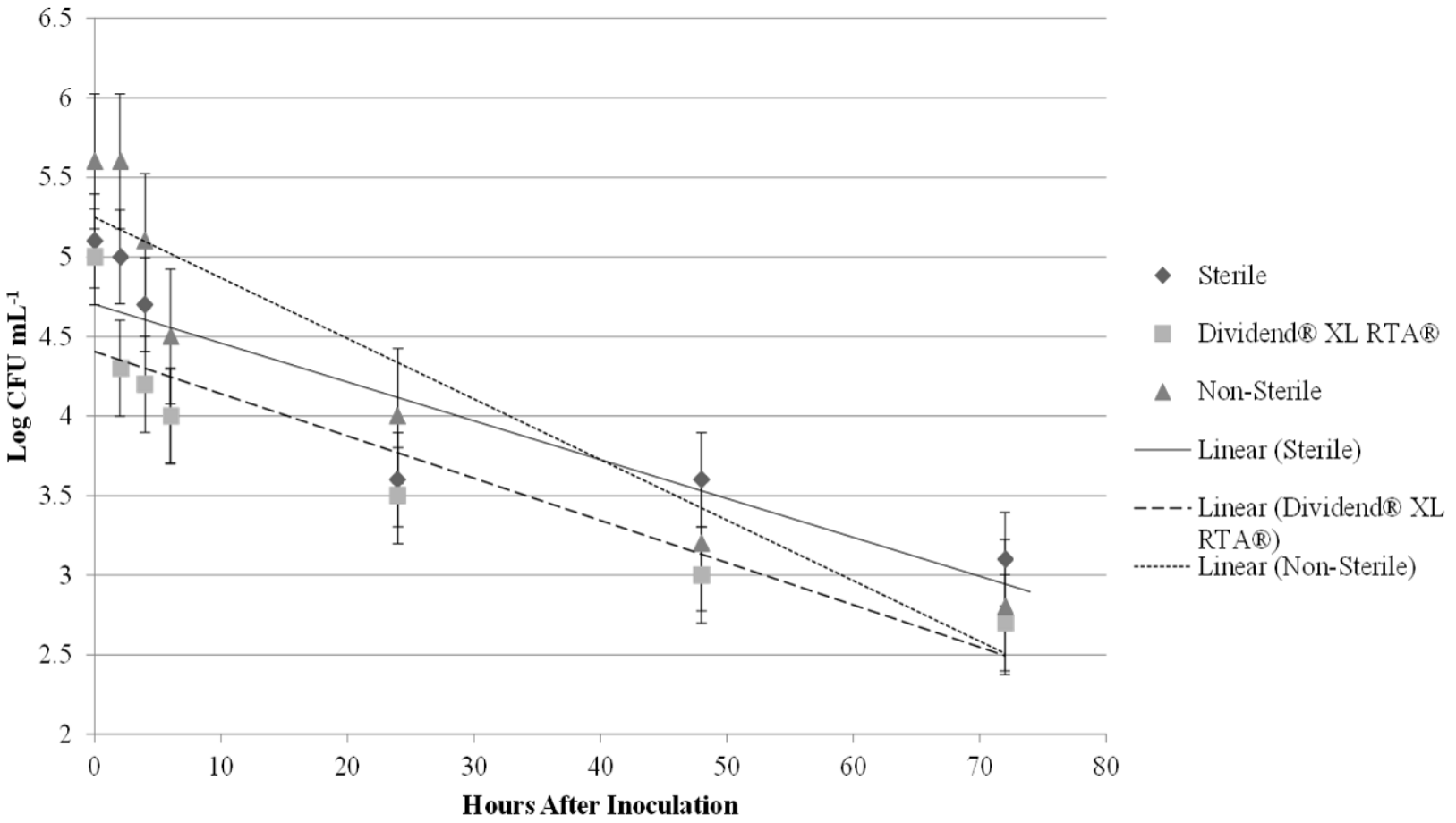


Figure 3.1 Recovery of *G. azotocaptans* inoculated onto wheat seeds treated with three seed treatments over time. The equation for the best fit line in each seed treatment is: sterile seed y=-0.35x+5.62, r^2=0.95; Dividend XL RTA seed y=-0.37x+5.36, r^2=0.97; non-sterilized seed y=-0.51x+6.50, r^2-0.97. n=84.

azotocaptans CFU (Table 3.2). Based on calculated Z values, *P* values were all above the 0.05 significance level (Appendix A).

The amount of *A. lipoferum* that was recovered at time zero from each of the seed treatments was $10^{4.98}$ CFU recovered off of sterile seed, equivalent to 83% recovery; $10^{4.82}$ CFU on Dividend® XL RTA® treated seeds, equivalent to 80% recovery; and $10^{4.79}$ CFU recovered from non-sterilized seeds, equivalent to 79% recovery (Table 3.3). Significant differences ($P<0.05$) were not found between the three seed treatments at time zero. However compared to the initial *A. lipoferum* application of 10^6 CFU mL^{-1}, significant ($P<0.05$) differences were found. Six hours after inoculation, there was approximately a 20% decrease in the amount of CFU recovered for all seed treatments compared to the CFU retrieved at time zero of *A. lipoferum.*

After time zero, as time passed, there was fewer CFU recovered off of sterilized seed compared to CFU recovered off of non-sterilized seed and Dividend® XL RTA® treated seed (Figure 3.2). However, the differences were not significant ($P<0.05$) until 24 h after inoculation. At 48 h and 72 h after inoculation, CFU were almost undetectable for *A. lipoferum* retrieved from sterilized seed. These values were under $10^{2.5}$ CFU mL^{-1}. The retrieval of *A. lipoferum* from Dividend® XL RTA® treated wheat seed was higher than the other two seed treatments at all dilution time points.

Based on the sterilized CFU data, the slope comparison for the sterile seed was constructed on data points from 0 to 24 h after inoculating. Slope comparisons for Dividend® XL RTA® and non-sterilized seed were constructed on data points from 0 to 72 h after inoculating. As was done with *G. azotocaptans,* equation 3.1 was used to compare slopes for CFU of *A. lipoferum* recovery on three seed treatments. Results showed that when the slope of sterilized seed was compared to either non-sterilized seed or Dividend® XL RTA® treated seed slopes, there was significant ($P<0.05$) differences between them (Table 3.4). The slope for non-sterilized seed compared to Dividend® XL RTA® showed the two slopes were similar.

3.5 Discussion

The immediate response of associative, non-symbiotic PGPB when inoculated on seeds or soil varies considerably depending on the bacteria, plant species, soil type, inoculant density, and environmental conditions of the soil and seed. In general, shortly after bacteria are

Table 3.2 Slope comparisons of CFU recovery of *G. azotocaptans* from wheat treated with three seed treatments.

Seed Treatment Comparison	***Z* value**	***P* value**
Non-sterilized / Dividend® XL RTA®	-2.16838	0.9821
Dividend® XL RTA® / Sterilized	-0.34695	0.6331
Non-sterilized / Sterilized	-2.52674	0.9941

* Indicates significant differences ($P<0.05$).

Table 3.3. Comparison of the initial application and recovery of *A. lipoferum* (CFU mL^{-1}) from three wheat seed treatments at 0 and 6 hours after inoculation. The percentage of recovery uses the initial application of 10^6 CFU mL^{-1} as 100%.

Treatment	Recovery at 0 h		Recovery at 6 h	
	Log CFU mL^{-1}	%	Log CFU mL^{-1}	%
Initial application on all treatments	6.00a†			
Sterile Seed	4.98b	83	3.64b‡	61
Dividend® XL RTA®	4.82b	80	4.53b	75
Non-sterilized Seed	4.79b	79	3.97b	66

†Means followed by the same letter are not significantly different ($P<0.05$) at time zero.
‡Means followed by the same letter are not significantly different ($P<0.05$) at 6 h after inoculation compared to the initial application.

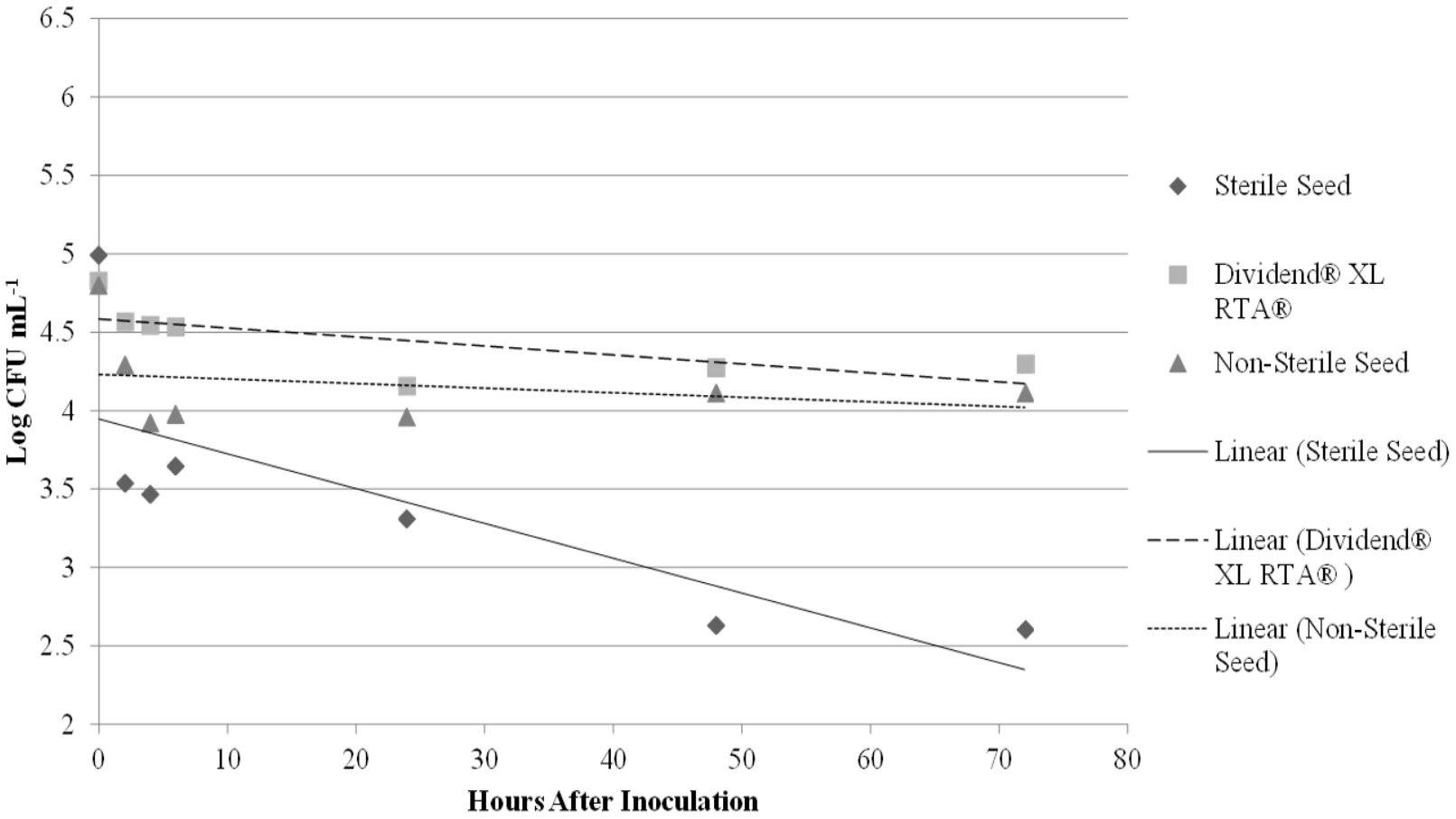


Figure 3.2. Recovery of *A. lipoferum* inoculated onto wheat seeds treated with three seed treatments. The equation for the best fit line in each seed treatment is: sterile seed y=-0.33x+4.75, r^2=0.78; Dividend® XL RTA® seed y=-0.09x+4.82, r^2=0.75; non-sterilized seed y=-0.08x+4.50, r^2=0.36. The value of n=78

Table 3.4 Slope comparisons of CFU recovery of *A. lipoferum* off wheat treated with three seed treatments.

Seed Treatment Comparison	*Z* value	*P* value
Non-sterilized / Dividend® XL RTA®	0.879138	0.1897
Dividend® XL RTA® / Sterilized	1.964096	0.0248*
Non-sterilized / Sterilized	2.248625	0.0123*

*Indicates significant differences ($P<0.05$).

introduced onto the seed or into the soil, the bacterial population declines progressively (van Elsas et al., 1986; Bashan and Levanony, 1988). Even when inoculated at large numbers initially (up to 10^7 CFU seed^{-1}), the number of associative bacteria rapidly decreases until in most cases 10^3 to 10^5 CFU g^{-1} plant root is reached (Jacoud et al., 1999; Burdman et al., 2000). From there the inoculated microorganisms must effectively colonize roots, survive and proliferate along growing plant roots over a considerable time period, in the presence of the indigenous microflora (Weller, 1988; Lugtenburg and Dekkers, 1999; Whipps, 2001). Seed environments are heterogeneous and unpredictable, even on a small scale (van Elsas and van Oberbeek, 1993). The inoculated bacteria sometimes cannot find an empty niche for survival except under sterilized environments, a condition which does not exist in large-scale agriculture. They must compete with other better-adapted native microflora and withstand predation (Bashan, 1998).

In this study, sterilized wheat was used as a control to compare to the non-sterilized seed. This was done to understand if there was any impact from native microorganisms on the seed coat of the non-sterilized seed on *A. lipoferum* or *G. azotocaptans* survival. However, this study did not enumerate the numbers of naturally occurring microorganisms on the seed coat. The seed was collected in March of 2008 and was stored in seed bags until used in the experiment, therefore native microorganism populations on the seed coat could have been reduced.

At time zero, all of the samples tested for the recovery of CFU had between $10^{5.08}$ and $10^{5.65}$ viable *G. azotocaptans* on the growth media. However, as time progressed, retrieval of viable bacteria from the seed surface became less for all wheat seed treatments. This result was anticipated. Even though there were different retrieval amounts of CFU from each seed treatment at each dilution time, slope comparisons confirmed that there was no difference from any seed treatment CFU recovery over time. Even though it was not significant, when recovery from sterilized seed and non-sterilized seed was compared, CFU from non-sterilized seed was higher. Therefore, competition from other microorganisms on the seed coat did not appear to be a major influencing factor reducing *G. azotocaptans* CFU.

Azospirillum lipoferum had comparable amounts of CFU recovered on all three seed treatments at time zero. Values were between $10^{4.79}$ and $10^{4.98}$ CFU. However, after time zero, there was a rapid decline in CFU retrieved from sterilized seed compared to recovery from the other two seed treatments. The reason for this rapid decline on sterile seed is unknown.

Fungicide seed treatments are frequently used to improve early plant emergence and to control the early attack by microbial pests. This strategy is familiar and useful in reducing seedling fatalities from seed-borne pathogens and seedling damping off agents (Phipps, 1984; Sinclair and Backman, 1989). In the past there have been studies that have been concentrated on effects of captan or thiram on *Azospirillum* spp. (Gallori et al., 1988; 1991; Di Ciocco and Caceres, 1997). However, captan and thiram are older fungicides and many steps have been taken with newer formulations of fungicides to make them safer on non-target microorganisms (Mary Leggett, personal communication).

Dividend® XL RTA®, currently a common fungicide used as a seed treatment for the control of seed and soil borne diseases on wheat in western Canada, was chosen to test for compatibility with *G. azotocaptans* and *A. lipoferum* (Ahmad and Khan, 2010; Saskatchewan Agriculture, Food and Rural Revitalization, 2010). However, these compounds should be specific for their targets, and have no effects on other soil microorganisms (Pereyra et al., 2009). Inoculant populations may be reduced when the wet inoculant is attached to the chemically coated seeds. Reductions could be due to the chemical in the fungicide, or even from the formulation properties (e.g., preservatives or colorants). Hence, even though we were using bacteria as inoculant, and a fungicide seed treatment, compatibility experiments are still important processes in developing inoculants. Results in this study showed that for both diazotrophs, cell survival was not hampered by Dividend® XL RTA®.

Most applicable studies with fungicides have only been done on *Azospirillum* spp such as: Cycon et al. (2006) demonstrated that N_2-fixing bacterial numbers augmented in accordance to increased levels of Tebuconazole in soil. Amongst 20 different types of pesticides, fungicides were the most toxic compounds to *A. brasilense* in *in vitro* growth (Gallori et al., 1991). Omar and Abd-Alla (1992) observed a progressive decrease in the growth of *A. brasilense* and *A. lipoferum* with increasing concentrations of a mixture of two thiocarbamates and copper oxychloride fungicides. Data suggests that some of the inhibitory effect on bacterial growth in many commercial fungicide formulations may be due to the presence of excipient compounds (e.g., colorants or powders; Pereyra et al., 2009).

On the other hand, Madhuri and Rangaswamy (2003) found that after 24 h of incubation in soils treated with recommended rates of fungicides (2.5 and 5.0 kg ha^{-1}) such as

monocrotophos, chlorphyrifos, mancozeb and carbendazim, *Azospirillum* populations reached a maximum stimulation at different fungicide doses. At higher fungicide concentrations (7.5 and 10.0 kg ha^{-1}) the pesticides exerted antagonistic interactions on the population of *Azospirillum* spp. Another study using applied seed dressing (Baytan, Vitavax, and Oxafun) reported no large effects on *Azospirillum* bacterium population under wheat, oat, and maize crops (Swędrzyńska and Sawicka, 2001).

In conclusion, there is not much compatibility research published on *G. azotocaptans* and *A. lipoferum* specifically, and no published data regarding compatibility of inoculants with Dividend® XL RTA® seed treatment.

Future research would be beneficial on having other inoculant carriers besides liquid formulations available for both diazotroph species. That would potentially reduce the bacterial losses from desiccation right after inoculation. There are also many other fungicide products used on western Canadian wheat that should be tested for compatibility with *G. azotocaptans* and *A. lipoferum* before they could be used as commercial inoculants. This is especially important due to the variety of responses (negative, neutral, and beneficial) from fungicide compatibility with diazotrophs that have been recorded in other studies.

This study was able to confirm that *G. azotocaptans* and *A. lipoferum* were able to be recovered from Dividend® XL RTA® treated seed, and no deleterious effects were observed from the fungicide on the diazotroph species. Competition with other microorganisms, which could have been on the wheat seed coat, were also not a concern to both diazotrophs survival on the seed.

4. IMPACT OF INOCULATION OF WHEAT WITH *GLUCONACETOBACTER AZOTOCAPTANS* AND *AZOSPIRILLUM LIPOFERUM* ON NITROGEN UPTAKE UNDER CONTROLLED GROWTH CHAMBER CONDITIONS

4.1 Preface

An understanding of the survival over time of *G. azotocaptans* and *A. lipoferum* as an inoculant on wheat seed was established in Chapter 3. These results were expanded into the present study to evaluate both diazotroph species inoculated onto wheat seed, *Triticum aestivum* (cv. Lillian), seeded into potted soil, and grown to maturity in a growth chamber. Performing this study in a growth chamber enabled the quantification of responses of wheat to diazotroph inoculation without external interferences. In addition, the influence of nitrogen (N) fertilization on N_2-fixation and N uptake was investigated.

4.2 Introduction

Rhizosphere soil is a "hot-spot" for microbial growth and major microbial activities (Sachdev et al., 2009). The growth of many microorganisms in the rhizospheric region depends on the root exudates released by the plants (Bais et al., 2006). Interactions between plants and microbes have been intensely studied; especially those microorganisms that benefit plant growth. Such free-living soil bacteria isolated from the rhizosphere of plants, which are beneficial for plant growth, are referred to as plant-growth-promoting-bacteria (PGPB) (Klopper et al., 1988; Wu et al., 1995; Bashan, 1998). Nitrogen fixers, also called diazotrophs, play a critical role in the plant ecosystem by reducing N_2 to NH_3 (Dilworth, 1974).

Nitrogen fixation is carried out by a diverse group of prokaryotes, bacteria and Archaea (Zehr et al., 2003). These include symbiotic N_2-fixing organisms such as *Rhizobium*, the obligate symbionts in leguminous plants, and non-symbiotic (free-living) organisms such as *Azospirillum*, *Azotobacter*, *Acetobacter*, and cyanobacteria. Diazotrophs can improve plant growth when they synthesize phytohormones and vitamins (Dobbelaere et al., 2003; El-Komy, 2005; Gray and Smith, 2005), improve nutrient uptake (Kennedy et al., 2004; Hoagland et al., 2008), enhance stress resistance, and solubilize inorganic P (Raj et al., 2003; Van Loon, 2007; Sachdev et al., 2009). Indirectly, PGPR are able to prevent the deleterious effects of pathogenic microorganisms, mostly through the synthesis of antibiotics and/or fungicidal compounds, through competition for nutrients by siderophore (an iron binding ligand) production or by the induction of systemic resistance to pathogens (Kloepper et al., 1998; Dobbelaere et al., 2003)

Wheat is one of the major crops cultivated in western Canada and worldwide, and its production is highly dependent on industrial chemically produced N fertilizers. As the human population grows, the increase in wheat consumed will present an economic challenge due to the impact of rising fossil energy costs on synthetic N fertilizer production, as well as an environmental challenge to maintain soil and water quality. Therefore, benefits from biological N_2-fixation (BNF) in wheat cultivation may prove to be very important from both an environmental and economic perspective and for sustainability (Dobereiner, 1995; Chelius and Triplett, 2000; Riggs et al., 2001; Kennedy et al., 2004)

Root exudate production is highest during the flowering stage of wheat compared to other stages. Hence, there are greater amounts of microbial biota in the rhizosphere and activity expected during this stage (Huddedard et al., 2000). Fertilization rates can influence both wheat life cycle (Charles et al., 2006) as well as microbial activity (Kennedy et al., 2004). Therefore, this study was conducted in a growth chamber to enable the quantification of N_2-fixation and uptake at three harvest times, as well as at three fertilizer rates without external interferences. Heads and stems were also analyzed separately at maturity to evaluate if any differences in plant parts arose.

4.3 Materials and methods

4.3.1 Preparation for planting

Soil was collected from a producer's field in the Moist Dark Brown soil zone. The previous year wheat was grown and the straw spread at harvest. Top soil was collected from approximately the 0 to 15 cm depth. Based on soil test analysis done by ALS Laboratory (Saskatoon, SK), the soil was a sandy loam and non-saline (Table 4.1). A conversion was done based on the texture and depth of the sample to convert units to $\mu g\ g^{-1}$ of potted soil (Diane Kemppainen, ALS Laboratory, personal communication). A total of 135 pots, 20.3 cm (diameter), were each filled with 2850 g of air dry soil. The soil was sieved to 5 mm before potting.

Soils hold different amounts of water depending on their texture and structure. The maximum amount of water a soil can hold before it is saturated and starts to lose water by gravity is known as field capacity (Meyer and Gee, 1999). The moisture holding capacity (MHC) of the soil was estimated as follows: four replicates of a 20 cm tall PVC pipe with a 5 cm diam were prepared with a coffee filter secured to the bottom. The pipes were packed with soil and the open end with filter paper was placed in a dish. The dish was filled with water and allowed to saturate the soil columns. After two days, the columns were removed from the dish and allowed to drain for 24 h. The water remaining in the total soil column was estimated to be at field capacity. The soil was air dried for five days in a drying room at 30°C, and weighed to determine the water content of the soil (Appendix B).

Table 4.1. Soil analysis for growth chamber soil.

Soil Characteristics	Measurement
pH	7
Electrical Conductivity	0.5 dS m^{-1}
Organic Matter	0.9%
N	3.2 µg g^{-1}
P	15.5 µg g^{-1}
K	141.8 µg g^{-1}
S	3.6 µg g^{-1}

$$MHC(\%) = \frac{[\text{wet weight soil (g)} - \text{dry weight soil (g)}]}{\text{dry weight soil (g)}} \text{x } 100 \qquad [4.1]$$

Pots were kept near 80% MHC by watering every two days during the experiment. The 80% MHC was achieved by weighing each pot and adding a volume of water to each pot to bring it back up to the proper weight during watering days.

4.3.2. Experimental design

The experiment was a completely randomized design with a factorial treatment of three fertilizer treatments; 0, 12.2, and 24.5 µg N g^{-1} of potted soil, three inoculant treatments (uninoculated control, *A. lipoferum*, and *G. azotocaptans*), and three harvest times (5-leaf, flag-leaf, maturity). Each treatment was replicated five times. The uninoculated control wheat was assumed to not fix N, and was therefore used as the non-fixing crop for the diazotroph treatments to be compared to.

4.3.3 Fertilizer

Granular urea fertilizer was dissolved in water and applied at the rate stated above to appropriate pots. Nitrogen from N_2-fixation was estimated using the ^{15}N isotope dilution method. Using ^{15}N-urea (10 atom %), 2 µg N g^{-1} was applied to all pots. (Appendix C).

In a 2 L flask, 1 mL of ^{15}N urea and 10 mL of 0, 12.2 or 24.5 µg N g^{-1} solution were added. Enough water was added to the flask to bring the volume up to 80% MHC for the soil (equivalent to 636 mL). The solution was stirred and slowly added to the corresponding pot.

4.3.4 Inoculant

Stock solutions were produced as they were for the lab experiment (section 3.3.1) where G5-4 medium at pH 7 was used for culturing *A. lipoferum* and G5-4 medium at pH 6 was used for culturing *G. azotocaptans.* Wheat seeds, cv Lillian, were inoculated with *A. lipoferum* or *G. azotocaptans*, or a control treatment of sterile water. The inoculants contained approximately $1x10^6$ CFU mL^{-1}. A 1 kg bulk wheat seed sample was inoculated with 10 mL kg^{-1} of each diazotroph species or water treatment. Each bag of seed was shaken vigorously until there was

even application of the seed treatment on the wheat seed. The bags were left open to air dry for 1 min then closed.

4.3.5 Seeding

Twelve wheat seeds with one of the three inoculant treatments were placed 2.5 cm below the soil surface of each pot. Nitrogen treatments (Section 4.3.3) were applied to the soil. Once plants reached the 2-leaf stage plants were thinned to four plants per pot.

The pots were placed in a growth chamber that was set to 16 h of light at 22°C and 8 h of dark at 15°C. At each watering period, pots were re-randomized.

4.3.6 Harvesting plants

Five replicates of each treatment were harvested at three times; 5-leaf (~40 days after planting), flag-leaf (~65 days after planting) and maturity (~102 days after planting). Above-ground plant parts were removed from the roots using scissors and stored in separate cloth bags. Shoots harvested at full maturity were separated into heads and stems.

Plant samples were dried in a drying room for five days at 60°C. Dry samples were weighed, and at mature harvest the number of wheat heads were counted and weighed. Mature plants were split, with two plants being analyzed as total plant material, and two plants separated into heads and stems for analysis. Separate head and stem analysis of wheat plants was done to see if there were different effects from the diazotrophs on each plant part. Plant parts were ground with a Wiley mill to pass through a 20-mesh sieve and then pulverized in a rotating ball mill in preparation for further analysis.

A solution of 75% ethanol was used to clean the mill between samples after vacuum cleaning. All samples were then run for total N and ^{15}N analysis at the University of Saskatchewan.

4.4 Nitrogen analysis

Nitrogen content of the plant tissues was determined using a LECO 2000 analyzer (LECO, St. Joseph, MI). Atom % ^{15}N was determined by mass spectrometry using a Costech

ECS4010 elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA), coupled to a Delta V mass spectrometer with Conflo IV interface (Thermo Fisher Scientific, Waltham, MA).

The ^{15}N isotope dilution method was used to quantify N biologically fixed by *A. lipoferum* and *G. azotocaptans*, and accumulated by wheat. This method involves the testing of an N_2-fixing crop and a suitable non-N_2-fixing control (Fried and Middelboe, 1977; Boddey et al., 1983; Rennie et al., 1983; Rennie and Dubetz, 1984; Reichardt et al., 1987), both grown in a substrate with homogenous ^{15}N enrichment. In this experiment, the wheat inoculated with the diazotroph strains are the N_2-fixing crop and wheat in the uninoculated control treatment was regarded as the non-N_2-fixing crop.

In the fixing plants, the $^{15}N/^{14}N$ ratio within plant tissues is lowered by the N_2-fixed from the unlabelled N in the atmosphere. Therefore the percentage of fixed N in the plant was determined according to Equation 4.2.

$$\%\text{Ndfa} = \left(1 - \frac{\text{atom}\,\%^{15}\,\text{N excess}_{(F)}}{\text{atom}\,\%^{15}\,\text{N excess}_{(NF)}}\right) \text{x}\, 100 \qquad [4.2]$$

Where %Ndfa is the percentage of N derived from the atmosphere; atom $\%^{15}N$ excess is the enrichment of the fixing (F) and non-fixing (NF) crops with the natural abundance ^{15}N of 0.3663 subtracted, respectively (Hardarson and Danso, 1990; Danso et al., 1993; Boddey et al., 1995; IAEA, 2001).

The amount of fixed N, expressed as $\mu g\ g^{-1}$, was determined using Equation 4.3, where, N yield is the amount of N uptake in plant parts or grain.

$$N_2 \text{ fixed } (g\,pot^{-1}) = \frac{\%\,\text{Ndfa}}{100} \text{ x N yield } (g\,pot^{-1}) \qquad [4.3]$$

4.5 Statistical Analyses

At each harvest time, data was analysed using analysis of variance (ANOVA) followed by Tukey's t-test ($P<0.05$) in SAS (JMP Version 8.0.2, SAS Institute Inc. Cary, NC, USA).

4.6 Results

4.6.1 Dry matter

Statistical analyses carried out for the three harvest times on the effect of inoculation and N fertilization on the dry weight of above-ground plant material showed that each harvest analysis had different factors that were significant ($P<0.05$; Table 4.2). The interaction between inoculant treatment and fertilizer level was significant ($P<0.05$) for dry matter yield at the 5-leaf and flag-leaf stage as well as for heads at maturity. Individually, fertilizer rates were significant ($P<0.05$) at the flag-leaf and mature harvest stages, as well as for heads and stems; and the inoculant treatment was significant ($P<0.05$) at the flag-leaf and mature harvest time, as well as for heads.

At the 5-leaf stage, wheat treated with *G. azotocaptans* with 24.5 μg N g^{-1} fertilizer had the highest dry matter yield of 1.1 g pot^{-1} (Figure 4.1a). This was significantly ($P<0.05$) higher when compared to unfertilized wheat treated with *A. lipoferum* or *G. azotocaptans*, and the uninoculated control fertilized with 12.5 μg N g^{-1}. At the flag-leaf harvest (Figure 4.1b), uninoculated wheat fertilized with 12.2 μg N g^{-1} had the highest accumulation of dry matter (11.0 g pot^{-1}). However, this value was not significantly different compared to all wheat treatments fertilized with 24.5 μg N g^{-1} or wheat inoculated with *A. lipoferum* with no fertilizer. Dry matter yield was lowest at the flag-leaf harvest for wheat plants inoculated with *G. azotocaptans* with no fertilization and with 12.2 μg N g^{-1} (7.4 and 7.1 g pot^{-1} respectively), as well as for wheat inoculated with *A. lipoferum* and 12.2 μg N g^{-1} (8.0 g pot^{-1}).

Stem analysis (Figure 4.1c) showed wheat inoculated with *G. azotocaptans* produced the most dry matter in stems and fertilized with 24.5 μg N g^{-1} (18.4 g pot^{-1}). This was significantly ($P<0.05$) higher than dry matter accumulation of mature stems of uninoculated wheat with no fertilization (11.9 g pot^{-1}). Wheat inoculated with *G. azotocaptans* at any fertilizer level was significantly higher than uninoculated wheat with no fertilizer. Analysis of heads showed that both uninoculated wheat and *A. lipoferum* inoculated wheat with 24.5 μg N g^{-1} (18.4 and 19.7 g pot^{-1} respectively) had significantly ($P<0.05$) higher dry matter compared to uninoculated wheat with 12.2 μg N g^{-1} and *G. azotocaptans* inoculated wheat with no fertilization.

Table 4.2. Analysis of variance of above-ground dry matter and number of heads produced at three harvest times of plants inoculated with three seed treatments (trt) at three fertilizer levels (fert). Plants at maturity were separated into total biomass, heads, and stems and analyzed.

Harvest Time		Source of Variance	Degrees of Freedom	Sum of Squares	F Ratio	Prob>F
Biomass	**Components at Maturity**					
5-leaf		trt	2	0.046	1.1516	0.3282
		fert	2	0.204	5.0409	0.0121*
		Trt*fert	4	0.397	4.9012	0.0031*
		Error	34	0.690		
Flag-leaf		trt	2	31.810	11.0132	0.0002*
		fert	2	35.427	12.2655	<0.0001*
		Trt*fert	4	19.387	3.3561	0.0203*
		Error	34	49.102		
Maturity	Total Biomass	trt	2	71.498	14.7963	<0.0001*
		fert	2	164.079	33.9553	<0.0001*
		Trt*fert	4	8.165	0.8449	0.5066
		Error	34	82.147		
	Heads	trt	2	48.999	9.7459	0.0005*
		fert	2	30.4022	6.0464	0.0057*
		Trt*fert	4	30.3188	3.0149	0.0313*
		Error	34	85.4780		
	Number of Heads	trt	2	27.610	1.511	0.2351
		fert	2	7.297	0.399	0.6738
		Trt*fert	4	96.537	2.642	0.0505
		Error	34	310.600		
	Stems	trt	2	76.301	19.3039	<0.0001*
		fert	2	49.856	12.6134	<0.0001*
		Trt*fert	4	15.597	1.9731	0.1209
		Error	34	67.195		

*significant interaction ($P<0.05$)

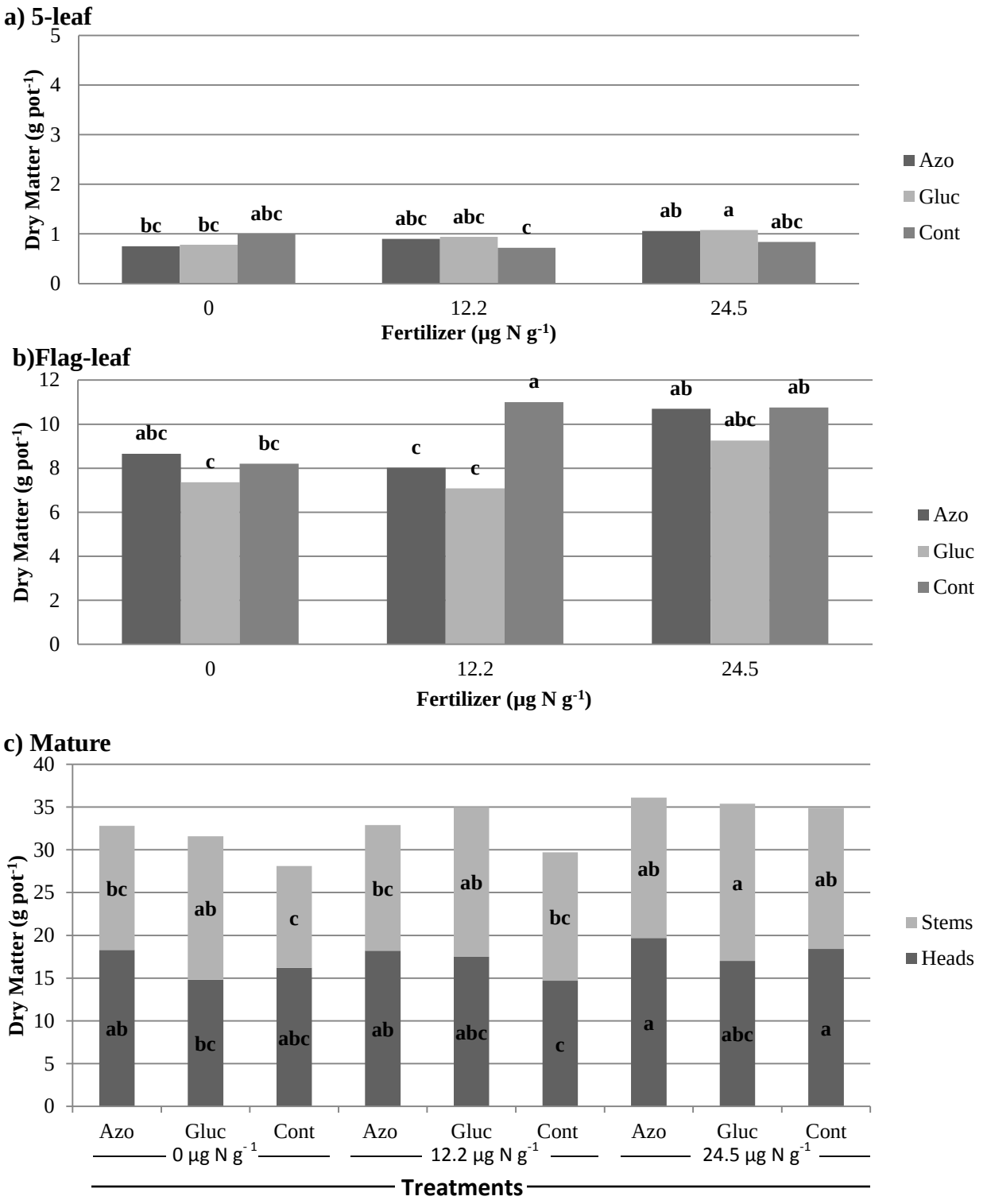


Figure 4.1 Dry matter accumulation of wheat at a) 5-leaf, b) flag-leaf, and c) maturity, where the interaction of inoculant treatments; uninoculated (Cont), *Azospirillum lipoferum* (Azo) or *Gluconacetobacter azotocaptans* (Gluc); and fertilization was significant. Means followed by the same lower case letter within a harvest stage and across all fertilizer levels are not different according to Tukey's t-test (P<0.05). At maturity means of a plant part followed by the same lower case letters are not different for total above ground biomass according to a Tukey's t-test (P<0.05).

A pattern emerged when dry matter accumulation was analyzed based on the effects of fertilizer alone (Figure 4.2a). At all harvest times, and for analysis of heads and stems, as fertilizer levels increased, so did the amount of dry matter accumulation. Therefore, fertilization of wheat with 24.5 µg N g^{-1} was significantly (P<0.05) higher than the other two fertilizer levels.

At maturity, wheat inoculated with *A. lipoferum* had significantly (P<0.05) higher dry matter production than the other two seed treatments (Figure 4.2b). This was also true for analysis of mature heads. However, when stems were analyzed, wheat inoculated with *G. azotocaptans* had significantly (P<0.05) higher dry matter than *A. lipoferum* and the uninoculated control.

4.6.2. Percent nitrogen derived from air

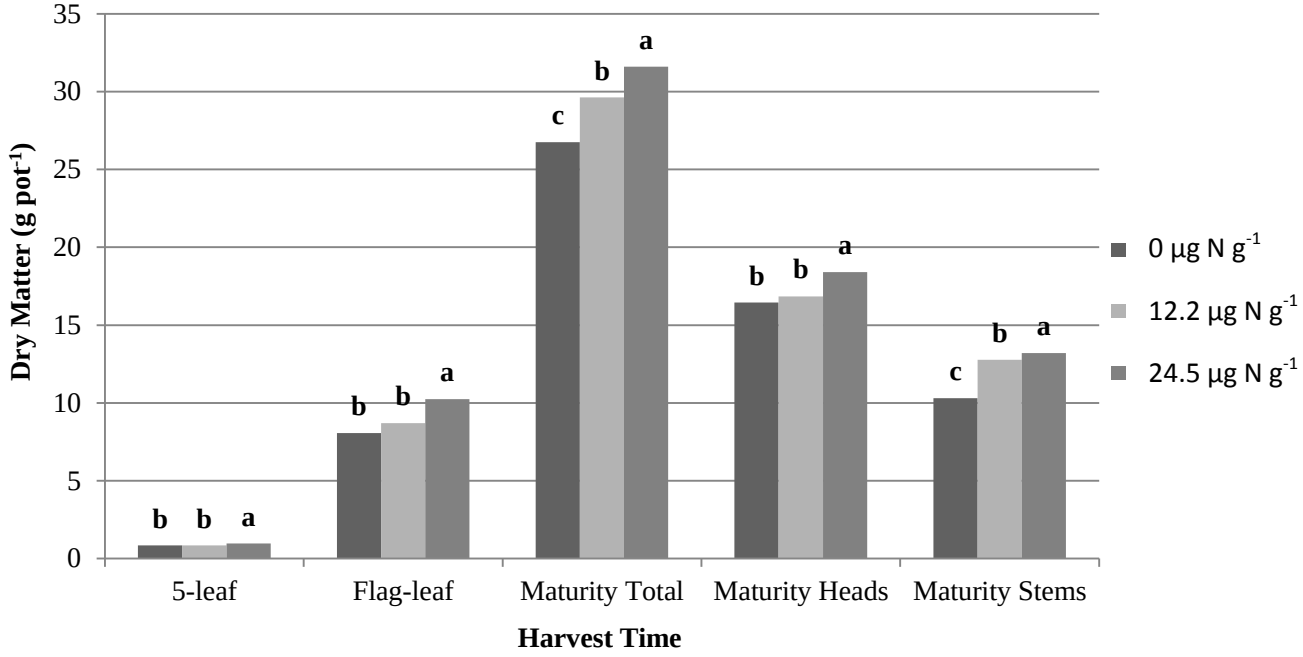
Values for %Ndfa were calculated using Equation 4.2, where the non-fixing treatment was the uninoculated wheat, at each corresponding fertilizer levels. Inoculation with either *G. azotocaptans* or *A. lipoferum* did not increase %Ndfa in wheat significantly (P<0.05) at any harvest stage. However, when results from the two diazotroph species were combined and analyzed, fertilizer had a significant effect (P<0.05) on %Ndfa at the 5-leaf and mature harvest stages, as well as when heads and stems were analyzed separately (Figure 4.3). Wheat plants that were fertilized with 12.2 and 24.5 µg N g^{-1} had the highest %Ndfa compared to non-fertilized plants.

4.6.3 Nitrogen uptake

Fertilizer level affected N uptake by wheat (Table 4.3) at the 5-leaf, flag-leaf, and mature harvest stages, as well as for mature heads analyzed separately. Seed treatment had a significant (P<0.05) effect on N uptake at 5-leaf and mature harvest stages, as well as for mature heads and stems analyzed separately.

Comparison of fertilizer treatments at the 5-leaf and flag-leaf harvest indicated additions of 24.5 µg N g^{-1} significantly (P<0.05) increased N uptake by wheat plants compared to the other two fertilizer treatments (Figure 4.4a). At maturity, wheat fertilized with 24.5 µg N g^{-1} had the highest amount of N uptake, followed by wheat fertilized with 12.2 µg N g^{-1}. Most of the N was

a) Fertilizer



b) Inoculant

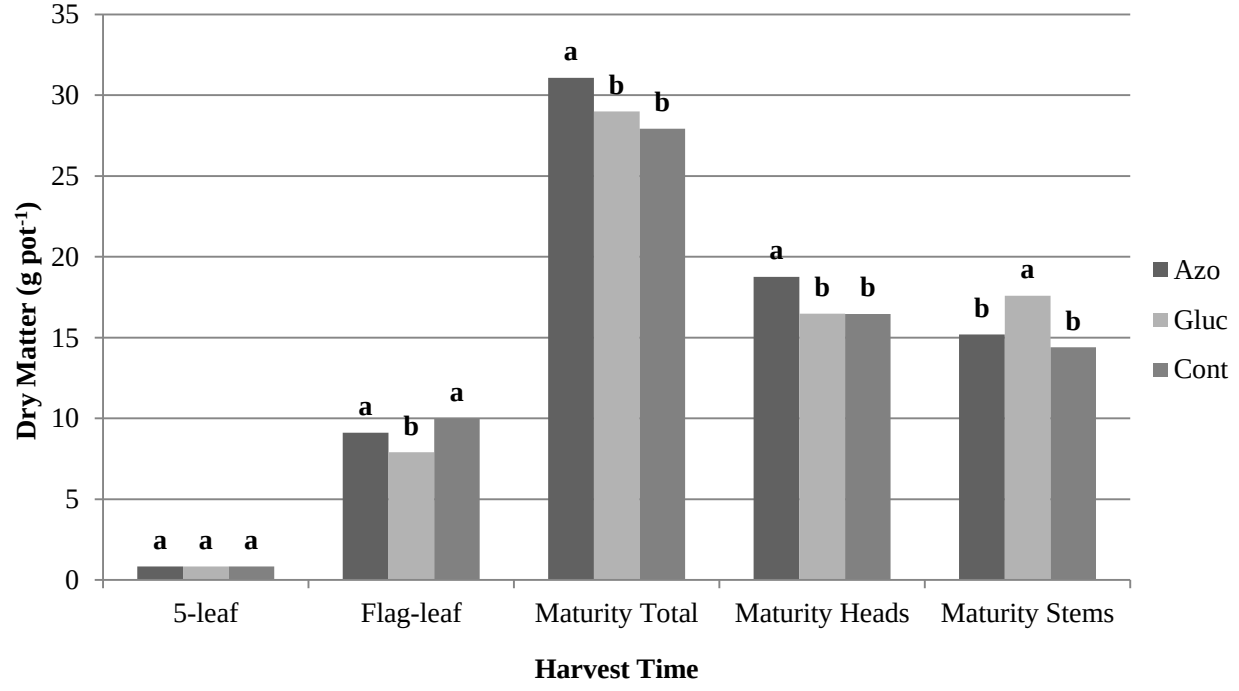


Figure 4.2 The amount of dry matter accumulation of wheat based on the effects from a) fertilizer levels and b) inoculant treatments; where Azo is *Azospirillum lipoferum*, Gluc is *Gluconacetobacter azotocaptans*, and Cont is uninoculated control. Within each harvest time or plant part, bars with the same letter are not significantly different according to Tukey's t-test (P<0.05).

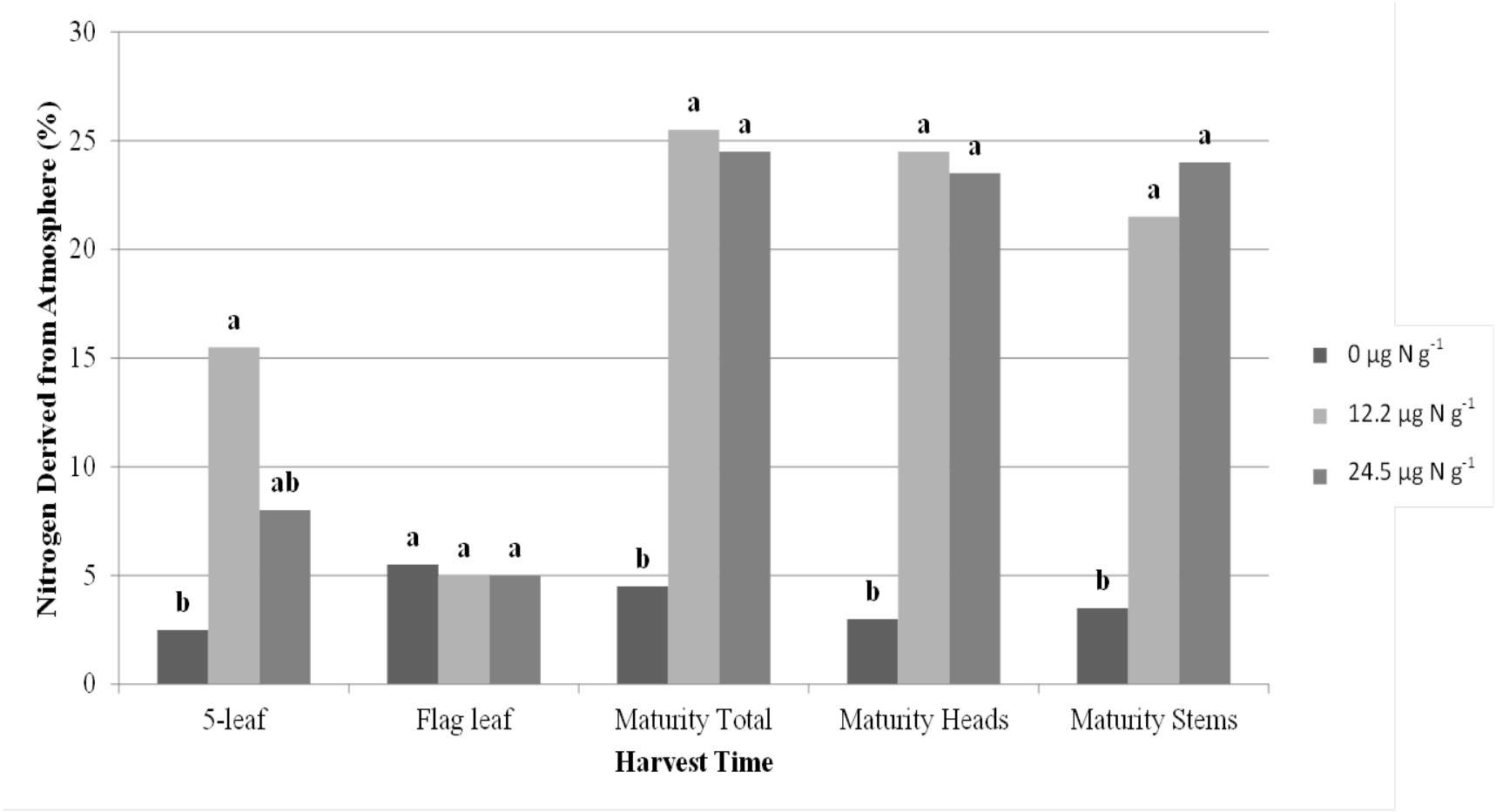


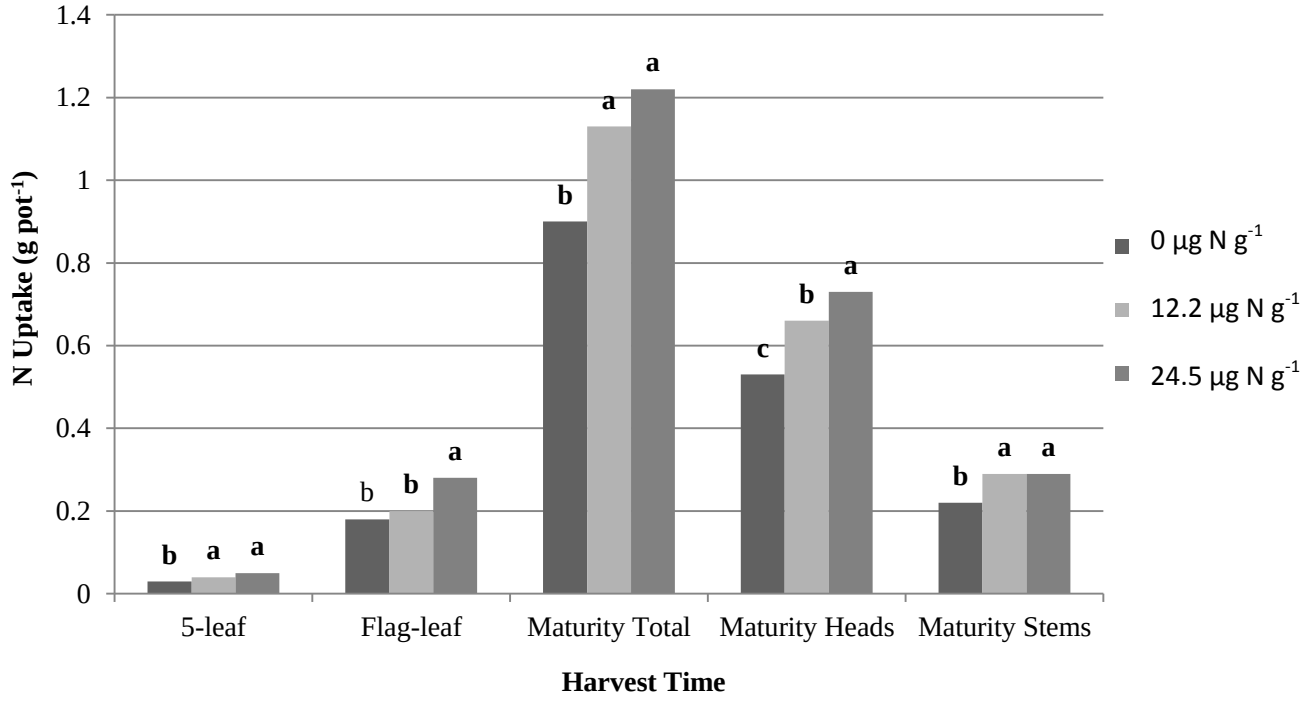
Figure 4.3. The effect of fertilizer rate on the percent nitrogen derived from atmosphere in wheat. Values are based on the combined analysis of amount of fixation from *A. lipoferum* and *G. azotocaptans* inoculation. Within a harvest time or plant part, means followed by the same letter at each harvest stage are not significantly different according to a Tukey's t-test ($P<0.05$).

Table 4.3 Analysis of variance of nitrogen uptake in wheat plants at three harvest times for plants inoculated with three seed treatments (trt) at three fertilizer levels (fert). Plants at maturity were separated into total plant material, heads, and stems and analyzed.

Harvest Time		Source of Variance	Degrees of Freedom	Sum of Squares	F Ratio	Prob>F
Biomass	Components at Maturity					
5-leaf		trt	2	0.0005	5.0279	0.0124*
		fert	2	0.0025	24.4745	<0.0001*
		Trt*fert	4	0.0003	1.3063	0.2880
		Error	33	1.3063		
Flag-leaf		trt	2	0.0100	2.9887	0.0646
		fert	2	0.0850	25.5076	<0.0001*
		Trt*fert	4	0.0020	0.3393	0.8494
		Error	32	0.0530		
Maturity	Total Biomass	trt	2	0.7848	21.1346	<0.0001*
		fert	2	0.6335	17.0598	<0.0001*
		Trt*fert	4	0.2185	2.9422	0.0378*
		Error	28	0.5199		
	Heads	trt	2	0.2828	28.2698	<0.0001*
		fert	2	0.2725	27.2422	<0.0001*
		Trt*fert	4	0.1196	5.9761	0.0009*
		Error	34	0.1701		
	Stems	trt	2	0.0534	9.7069	0.0005*
		fert	2	0.0517	9.3932	0.0006*
		Trt*fert	4	0.0228	2.0744	0.1059
		Error	34	0.0936		

*significant interaction ($P<0.05$)

a) Fertilizer



b) Inoculant

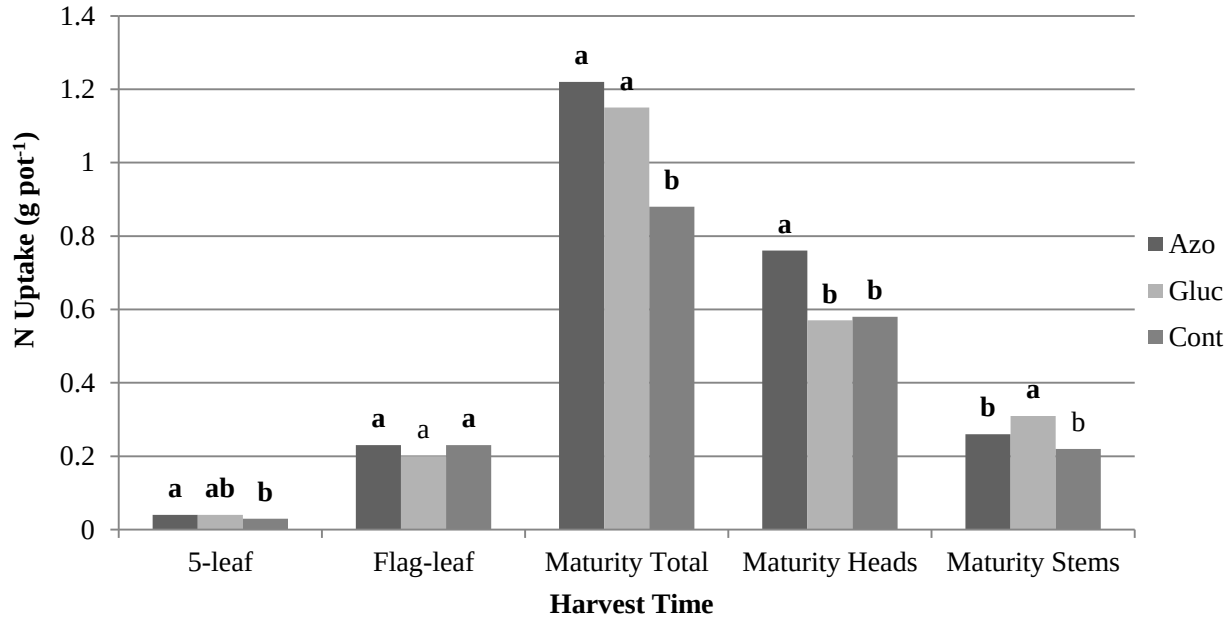


Figure 4.4 The amount of nitrogen uptake in wheat plants at three harvest times, based on the influence of a) fertilizer levels and b) wheat inoculants; where Azo is *Azospirillum lipoferum*, Gluc is *Gluconacetobacter azotocaptans*, and Cont is uninoculated control. Within a harvest time or plant part, means followed by the same letter are not significantly different according to a Tukey's test ($P<0.05$).

found in the heads of wheat plants. The lowest N uptake was found for wheat plants with no fertilizer additions.

At the 5-leaf harvest, *A. lipoferum* inoculated wheat took up significantly (P<0.05) more N compared to the uninoculated wheat (0.04 and 0.03 g pot^{-1}, respectively) (Figure 4.4b). At the mature harvest stage, both *A. lipoferum* and *G. azotocaptans* inoculated wheat had significantly (P<0.05) higher N uptake than the uninoculated control (1.22, 1.15 and 0.88 g pot^{-1}, respectively). Mature heads and stems were also analyzed separately. *Azospirillum lipoferum* inoculated wheat had the highest N uptake (0.76 g pot^{-1}) into heads compared to *G. azotocaptans* inoculated wheat and the uninoculated control (0.57 and 0.58 g pot^{-1}, respectively). Conversely, *G. azotocaptans* inoculated wheat showed the highest uptake of N (0.31 g pot^{-1}) for stems compared to *A. lipoferum* inoculated wheat and the uninoculated control (0.26 and 0.22 g pot^{-1}, respectively).

4.6.4. Amount of nitrogen fixed

Values for the amount of N-fixed (g pot^{-1}) in wheat inoculated with either *A. lipoferum* or *G. azotocaptans* were calculated using Equation 4.3, where the amount of N-fixed depended on the %Ndfa and N uptake of the wheat plants. Fertilizer had a significant effect (P<0.05) on accumulation of fixed N at the 5-leaf and mature harvest stages as well as for heads and stems (Table 4.4). Inoculant treatment was only significant (P<0.05) for the analysis of heads.

Fertilization rates of 12.2 or 24.5 µg N g^{-1} resulted in the accumulation of the most atmospheric N fixed in the inoculated wheat plants (Figure 4.5a). Significantly (P<0.05) less fixed N was taken up in wheat where no fertilizer N was applied for all sampling times except for the flag-leaf harvest, where no differences were observed. Wheat inoculated with *A. lipoferum* had significantly (P<0.05) higher fixed N in heads compared to the wheat inoculated with *G. azotocaptans* (Figure 4.5b).

4.7 Discussion

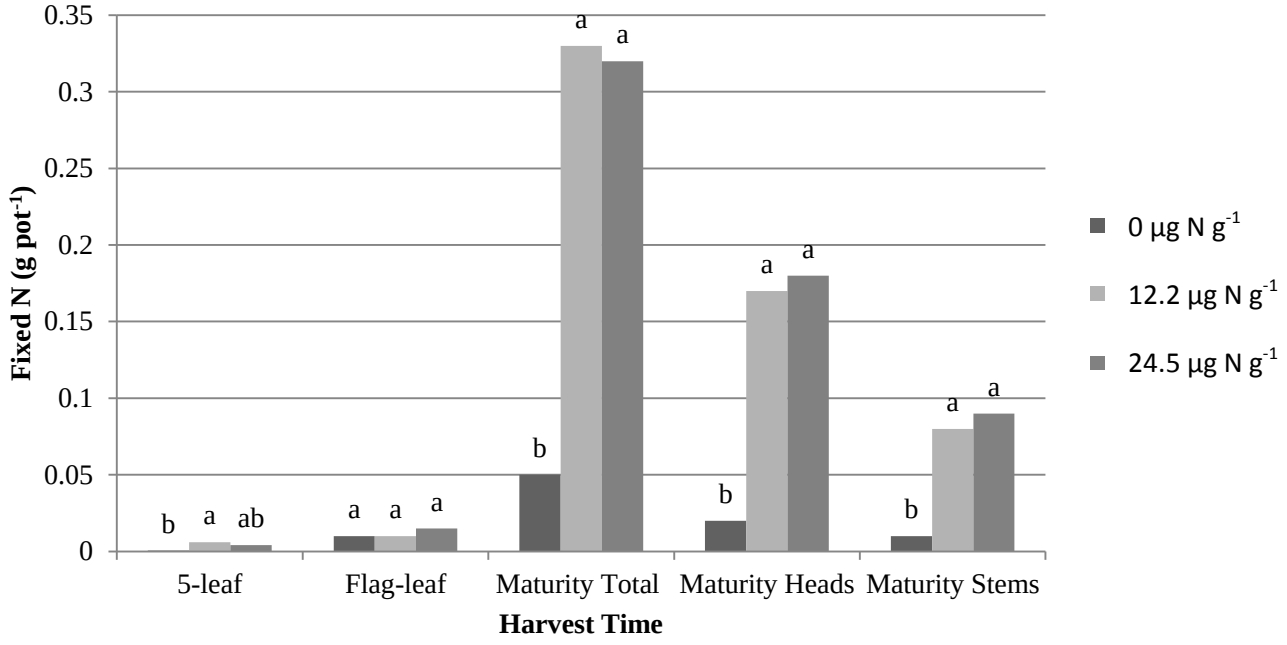
In this study two previously isolated bacteria from the corn rhizosphere, were tested for potential benefits to wheat growth. These diazotroph species, *A. lipoferum* and *G. azotocaptans*, were examined for their effect on plant growth and N uptake on wheat under growth chamber

Table 4.4 Analysis of variance of atmospheric fixed nitrogen in wheat plants at three harvest times for plants inoculated with three bacterial treatments (trt) at three fertilizer levels (fert). Plants at maturity were separated into total plant material, heads and stems and analyzed.

Harvest Time		Source of Variance	Degrees of Freedom	Sum of Squares	F Ratio	Prob>F
Biomass	Components at Maturity					
5-leaf		trt	1	0.00001	1.0486	0.3170
		fert	2	0.00012	5.3160	0.0131*
		trt*fert	2	0.00001	0.5967	0.5593
		Error	22	0.00025		
Flag-leaf		trt	1	0.00012	1.0357	0.3210
		fert	2	0.00012	0.5161	0.6046
		trt*fert	2	0.00007	0.2907	0.7508
		Error	20	0.00239		
Maturity	Total Biomass	trt	1	0.00679	1.0372	0.3246
		fert	2	0.35508	27.0857	<0.0001*
		trt*fert	2	0.00410	0.3134	0.7357
		Error	15	0.09832		
	Heads	trt	1	0.01226	7.3160	0.0133*
		fert	2	0.16127	48.1113	<0.0001*
		trt*fert	2	0.00545	1.6287	0.2200
		Error	21	0.03519		
	Stems	trt	1	0.00154	1.4263	0.2445
		fert	2	0.03803	17.6139	<0.0001*
		trt*fert	2	0.00089	0.4158	0.6647
		Error	23	0.02483		

*significant interaction ($P<0.05$)

a) Fertilizer



b) Inoculant

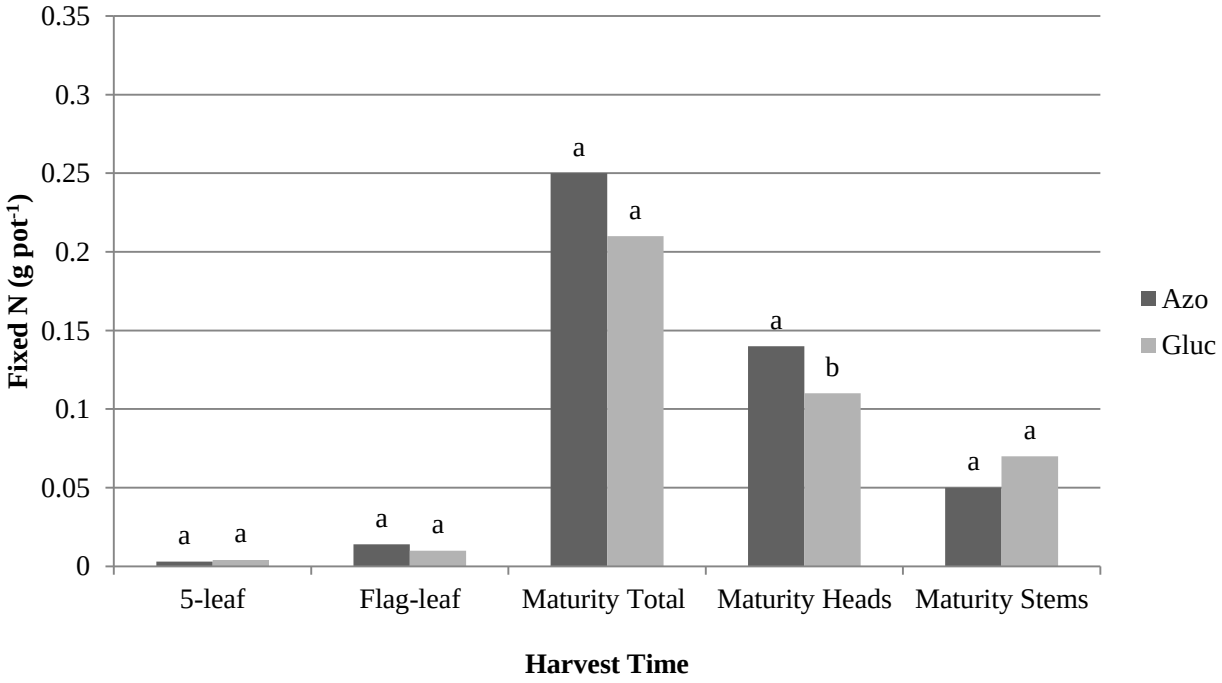


Figure 4.5 The effect of a) fertilizer rate and b) inoculant on the amount of atmospheric fixed nitrogen in wheat plants, where Azo is *Azospirillum lipoferum* and Gluc is *Gluconacetobacter azotocaptans.* Within a harvest time or plant part, means followed by the same letter are not significantly different according to a Tukey's t-test (P<0.05).

conditions using the ^{15}N dilution technique. This technique was used to quantify N_2-fixation based on the fact that it has been used widely for quantification of biologically fixed N in legumes as well as non-legumes (Urquiaga et al., 1992; Wu et al., 1995; Shrestha and Ladha, 1996; Boddey et al., 2000; Somado and Kuehne, 2006).

Inoculation of wheat with *G. azotocaptans* significantly inhibited dry matter at the flag-leaf stage of plant growth when no or low amounts of fertilizer were added to the pots (0 and 12.2 μg N g^{-1}). Furthermore, *G. azotocaptans* inoculated wheat produced significantly less dry matter than the other two seed treatments. There have been other studies where declines in yield were associated with inoculation (Nguyen et al., 2002). In contrast, Diaz-Zorita and Fernandez-Canigia (2008) found that positive effects of inoculation on early growth of plants not always translated into increased yields. Diazotrophs such as *Gluconacetobacter* may be more restricted in the range of plants they can be associated with because of special nutritional needs such as high sugar concentrations (Han and New, 1998; Kennedy et al., 2004). In this study, as wheat matured, the inoculant became more beneficial to plant growth. At maturity, *G. azotocaptans* inoculated wheat had higher dry matter values which was significantly (P<0.05) higher compared to the uninoculated control. Analysis of mature stems showed that *G. azotocaptans* treated wheat produced more dry matter than the other two treatments.

Azospirillum lipoferum increased dry matter production in wheat at maturity and in wheat heads significantly (P<0.05) over the other two seed treatments. Wheat inoculated with *A. lipoferum* may benefit the plant by fixing N, particularly at later stages of growth when the need for N increases during flowering and seed formation (Neyra and Dobereiner, 1977). A study by Santa et al. (2004) illustrated greater wheat grain accumulation, caused by inoculation with *Azospirillum* spp., due to a larger amount of N made available for the plant during flowering and grain filling. Significant increases in dry weight of *Pennisetum* and *Panicum* have been reported in field experiments with a medium-level of N fertilization (Smith et al., 1976), and results presented by Cohen et al. (1980) and Rai and Gaur (1982) also indicate that *Azospirillum*-grass associations work best with modest initial levels of N in the soil. The results presented here agree with this previous work, since additions of 12.2 and 24.5 μg N g^{-1} promoted dry matter production of wheat inoculated with *A. lipoferum*.

Quantification of biologically fixed N in legumes or grasses has been a very important factor in determining the overall benefit of this process to the cropping system (Malik et al., 1997). Techniques based on ^{15}N dilution are versatile and can be adapted to various experimental conditions (Roger and Ladha, 1992; Boddey et al., 1998). The ^{15}N isotope dilution technique has been used for quantifying associative N_2-fixation in wheat (Lethbridge and Davidson, 1983a; Rennie et al., 1983; Rennie and Rennie, 1983; Malik et al., 1997; Iniguez et al., 2004), maize (Rennie, 1980), rice (Ventura and Watanabe, 1983; Shrestha and Ladha, 1996), sugarcane (Ruschel et al., 1975; Boddey et al., 1998) and other grasses (Boddey et al., 1983; Boddey and Victoria, 1986; Malik et al., 1987). It is also the best method for demonstrating amounts of fixed N taken up by the total plant (Fried et al., 1983; Rennie and Rennie, 1983).

The calculation of %Ndfa was done at each separate fertilizer level using the uninoculated control treatment as the non-fixing control plant. Based on these findings, and results from a study by Rennie and Thomas (1987), it appears that the ability to incorporate atmospheric N into the plant, following inoculation of the wheat plant and the rhizosphere with diazotrophic bacteria, is a relatively common trait among Canadian wheat cultivars in the field. Mature wheat inoculated with either *A. lipoferum* or *G. azotocaptans*, and with fertilizer additions of 12.2 or 24.5 μg N g^{-1} had the highest %Ndfa (25.5%). At this harvest stage there was considerably more fixation occurring than the two earlier harvests, which is consistant with dry matter production. Fertilizer additions of 12.2 and 24.5 μg N g^{-1} increased %Ndfa for both diazotroph strains above that of the unfertilized plants as well as uninoculated controls; therefore it was concluded that urea supplied to the plants did not negatively affect N_2-fixation.

Early harvest assessment of %Ndfa was variable. A similar study showed a similar variability in inoculation with *A. brasilense*, where in one year *A. brasilense* averaged 15.5 %Ndfa, whereas in the next year, higher soil N levels reduced this value by approximately one-half (7.5 %Ndfa; Rennie and Thomas, 1987). In a study based on rice inoculated with different strains of *Azospirillum*, 6 to 27 %Ndfa was reported where the highest contribution from BNF was equivalent to 120 μg N g^{-1} and the lowest contribution was 29 μg N g^{-1}. Other pot and field studies have shown that N_2-fixation in cereals inoculated with *Azospirillum* is very low. For instance, in pot experiments with wheat using the ^{15}N isotope dilution method, no contributions from BNF were detectable (Lethbridge and Davidson, 1983b). Here it was shown that 2 to 26%

of the N in above-ground wheat plant material was derived from fixation by the inoculated bacterial species, but was dependant on the time of plant harvest and amount of fertilizer applied.

As fertilizer levels increased, so did the amount of N uptake by wheat. At all harvest times, and for heads and stems, wheat fertilized with 24.5 μg N g^{-1} had significantly more N uptake compared to plants that were not fertilized. This was an expected result because there was more available N for the plant to access and growth was stimulated.

In other studies, wheat inoculated with rhizobacteria increased grain N-yield and total N yield of plants (Salantur et al., 2006). Foxtail millet (*Setaria italic*) inoculated with *A. lipoferum* increased shoot N uptake 14 to 30% over control plants (Rao and Charyulu, 2005). Another experiment examined wheat seedlings inoculated with diazotrophs harvested at the 5-leaf stage and analyzed for N uptake. Values ranged between 38.1 mg and 45.5 mg total N (Micanovic et al., 2006). Rai and Gaur (1988) reported an increase in N uptake by wheat inoculated with *Azotobacter* or *Azospirillum* which they attributed to N_2-fixation. An earlier study reported that *Azospirillum* associated with the roots of grasses may benefit the plant both by producing growth hormones and by N_2-fixation, particularly at later stages of growth when the plant`s need for N increases during flowering and seed formation (Neyra and Dobereiner, 1977).

As was seen with dry matter accumulation, inoculation of wheat with *G. azotocaptans* showed significantly lowed N uptake at the flag-leaf stage compared to the other two seed treatments. The reduced plant material at this growth stage could have reduced the amount of plant root exudates available for bacteria to feed on (Walker et al., 2003; Kennedy et al., 2004). The inability of the host plant to release carbon (C) in the rhizosphere can be a significant constraint in the development of associative N_2-fixing systems (Anyia et al., 2009).

Nitrogen uptake in heads of mature plants showed that values of wheat inoculated with *A. lipoferum* were significantly ($P<0.05$) higher than the other two seed treatments. More N accumulation in wheat heads can lead to an increase in protein content (Campbell et al., 1977), which in turn could help increase the value of the grain. Diazotroph fixation and translocation into wheat heads could also benefit N-use efficiency (Roy et al., 2002), reducing the losses from denitrification and leaching associated with adding higher levels of N fertilizer, or using split applications of N, to increase protein levels during growth and maturity.

Stem analysis showed that *G. azotocaptans* is able to transfer more fixed N to the stems of plants. Again, there was a similarity to amounts of dry matter in mature stems. This outcome could indicate that using *G. azotocaptans* as an inoculant in wheat increases plant production of leaves potentially increase photosynthesis, and increasing tillers which could benefit the yield of the plant. Also, the increase in N uptake in stems could influence the amount of N returned back to the soil in straw at harvest.

In conclusion, the diazotroph species were able to fix atmospheric N that became available for uptake by wheat plants. Fertilization benefited N_2-fixation, where fertilizer rates of 12.2 and 24.5 µg N g^{-1} resulted in the highest amounts of %Ndfa, N uptake, and amount of N fixed in plants at maturity. When mature plants were separated into heads and stems and analyzed, N uptake and fixed N from *A. lipoferum* became located more in heads of the plants, and N uptake and fixed N from *G. azotocaptans* became located more in stems of the plants.

Further experiments to see if *A. lipoferum* and *G. azotocaptans* were compatible together would be of interest. If the two inoculants were compatible together, they could potentially be combined and dually inoculated on wheat plants. Therefore, benefiting all areas of plant growth.

5. IMPACT OF *GLUCONACETOBACER AZOTOCAPTANS* AND *AZOSPIRILLUM LIPOFERUM* INOCULATION OF WHEAT ON GROWTH, NITROGEN FIXATION AND NITROGEN UPTAKE UNDER FIELD CONDITIONS

5.1 Preface

In Chapter 4 we saw that nitrogen (N) fertilizer did not hinder *A. lipoferum* or *G. azotocaptans* growth or their ability to fix atmospheric N. Also, *A. lipoferum* increased dry matter production and N uptake at maturity and in wheat heads, whereas *G. azotocaptans* increased dry matter production and N uptake at maturity in stems of wheat plants. Plant impacts on rhizosphere microbial communities under controlled environmental conditions however, may not correlate with those that occur under field conditions. This is particularly true with diazotroph species that have regularly shown higher N_2-fixation in warmer climates, but have variable results in cool climate regions. In this field study, diazotrophs isolated from a cooler Canadian climate were inoculated onto wheat and grown in western Canada where N_2-fixation and N uptake were monitored. In addition, the influence of N fertilization on N2-fixation and N uptake was investigated.

5.2 Introduction

The presence of the root defines the rhizosphere, while the intimate interactions between the plant and soil biota within the soil habitat characterize the rhizosphere. Root exudates are the substrate or fuel for the intense microbial (bacteria, fungi, algae, protozoa, nematodes and arthropods) activity within the rhizosphere. Thus, it is the quantity and quality of the exudates and condition of the soil habitat that will determine the colonization potential of the rhizosphere (Lugtenberg et al., 2002). Each plant species leaks a unique carbon (C) and N signature of carbohydrates, amino acids and organic acids that determines the primary colonizers of the microbial community (Jones et al., 2009). The composition of the exudates also affects the availability of nutrients in the immediate vicinity of the roots, influencing the establishment and growth of the plant. An increase or decrease in the nutritional status of the plant can further alter the quality of the exudates, affecting the microbial diversity and populations in the rhizosphere. Root biomass and architecture, fertilizer type, tillage and cropping history also affect root growth and patterns of exudation and the populations and diversity of rhizosphere colonizing organisms. Together, these factors determine whether rhizosphere interactions and processes will have a positive, neutral, or beneficial effect on plant growth. Microbial inoculants placed with or in the immediate proximity of the seed must be able to successfully establish and multiply along the growing root, and integrate into the rhizosphere community, or alter and/or overwhelm the community to give the desired growth effect (Hnatowich, 2000).

Conducting experiments in a controlled environment allows the control of certain environmental conditions. This enables the quantification of a response to a determined stress, biotic or abiotic, without existing external interferences (Villora et al., 2004). Experiments under field conditions, however, seek to approach the real situations faced by farmers, thereby providing a better prediction of how the test application(s) perform under adverse environments, and finally, improving economic benefits. Presumably, results from these trials can then be extended to farmers (Mulongoy et al., 1992).

It must be clearly demonstrated that an inoculant biofertilizer is effective in improving crop yield, and in reducing the need for chemical fertilizers. Uptake of fixed N into shoots of plants has been demonstrated using $^{15}N_2$-enriched atmospheres (Giller et al., 1984; James, 2000; Bhattacharjee et al., 2008). In this field study, potential N_2-fixation rates were measured by ^{15}N

fertilizer incorporation into soil. This method has been widely applied (Brouzes et al., 1969; O'Toole and Knowles, 1973; Montoya et al., 1996). The purpose of this experiment was to evaluate the effects of *G. azotocaptans* and *A. lipoferum* inoculated onto wheat seeds on plant growth, N_2-fixation, and N uptake under field conditions.

5.3 2008 field season

5.3.1 Site locations

There were four sites in the 2008 growing season located near Langham, Loreburn, Saskatoon, and Vanguard, Saskatchewan. Locations were selected on the basis of soil available N levels, representation in different soil climatic zones, and previous cropping history. Soil sampling was done in the spring of each cropping season, and analysis was conducted by ALS Laboratory Group (Saskatoon, SK). Site locations, soil characteristics and available nutrient levels are described in Table 5.1.

5.3.2 Agronomic procedures

Wheat (*Triticum aestivum* cv Lillian) was seeded directly into standing cereal or canola stubble. Lillian wheat was chosen because of its solid stem characteristics. Areas of Saskatchewan can be prone to wheat damage from wheat stem sawfly. The solid stem deters the insect from laying eggs in the stem. Seeding dates ranged from May 16 to May 21, 2008. Soil moisture at the time of seeding was good. Wheat was seeded at a rate of 90 kg ha^{-1}.

Plots were seeded with a custom made air-drill manufactured by Black and White Machinery (Asquith, SK). Seed was metered using a Monosem vacuum metering system (Edwardsville, KS, USA), which holds the seed gently onto the holes of the seed disc. As the seed disc turns, seeds are ejected off the plate and seed is dropped at a constant rate. Stealth (Flexicoil, Saskatoon, SK) sideband double shoot openers were used with a 20 cm row spacing between seed rows. Seed was placed 2.5 cm above and 2.5 cm to the side of the fertilizer. Wheat was seeded 2.5 cm deep, directly into standing stubble.

Table 5.1 Location, soil characteristics, sample depth and available nutrients of the 2008 field sites.

Location	Field Information	Soil Characteristics	Sample Depth (cm)	Nutrient Levels (kg ha^{-1})			
				N	P	K	S
Langham	SE 19-39-9 W3 Black Soil Zone Previous crop oats Baled stubble Continuous cropping	Loam soil pH 6.7-7.4 Non Saline 3.4% OM	0-15 15-30	6.72 8.96	5.6	>600	11.2 10.08
Loreburn	NE 24-26-5 W3 Dark Brown Soil Zone Previous crop wheat Spread stubble Continuous cropping	Clay Loam soil pH 7.4-8.2 Non Saline 3.5% OM	0-15 15-30	10.08 3.36	25.76	>540	31.36 13.44
Saskatoon	SW 34-36-6 W3 Moist Dark Brown Soil Zone Previous crop canola Spread stubble Continuous cropping	Clay Loam soil pH 6.4-7 Non Saline 4.7% OM	0-15 15-30	15.68 10.08	29.12	>540	32.48 13.44
Vanguard	SW 17-11-9 W3 Brown Soil Zone Previous crop wheat Spread stubble Non-continuous cropping	Clay soil pH 8.1 Non Saline	0-30	22.4	15.68	>1020	23.52

All plots received a seed placed application of 24 kg ha^{-1} of 0-45-0 (triple superphosphate) fertilizer. An application of glyphosate prior to seeding sites was performed for non-selective weed control. In-crop weed control was accomplished by applying a foliar application of clodinafop-propargyl for grassy weeds, combined with MCPA, mecoprop-p and dicamba for broadleaf weeds. Desiccation at harvest was performed with diquat. All pesticides were applied at the manufacturer's recommended rates. Weeds that were not removed chemically were removed manually at various times throughout the growing season.

5.3.3 Inoculation

The inoculants used in the field trials were *G. azotocaptans* and *A. lipoferum*. These strains were produced at Novozymes BioAg (Saskatoon, SK) at the beginning of May using the same procedure described in section 3.3.1. Strains were stored on site from 4 d to 20 d, until needed at seeding. Application of the inoculant to the seed occurred in the field just prior to seeding.

The inoculants were applied at a rate of $1.07x10^9$ CFU *G. azotocaptans* per gram of seed, and $1.05x10^9$ CFU *A. lipoferum* per gram of seed. The inoculants and seed samples were combined in plastic bags in the field and shaken rigorously until there was even application of inoculant on the seed. The control treatment contained the same volume of water as the inoculant treatments.

5.4 2009 field season

5.4.1 Site locations

There were four site locations during the 2009 growing season, located at Saskatoon, Langham, Vanscoy, and Delisle, Saskatchewan. A fifth site located in Cabri, Saskatchewan was added when some inoculant storage problems arose, compromising the inoculant viability. Details are discussed in Section 5.4.3. Site selection was based on the same criteria as in 2008. ALS Laboratory Group analyzed the soil samples which are described in Table 5.2.

Table 5.2 Location, soil characteristics, sample depth and available nutrients of the 2009 field sites.

Location	Field Information	Soil Characteristics	Sample Depth (cm)	Nutrient Levels (kg ha^{-1})			
				N	P	K	S
Langham	NE 8-39-9 W3 Black Soil Zone Previous Crop Wheat Spread Stubble Continuous cropping	Loam soil pH 7.1-7.5 Non Saline 4.2% OM	0-15 15-30	14.56 7.84	11.2	631	>48 >48
Delisle	NW 23-33-9 W3 Dark Brown Soil Zone Previous Crop Canola Spread Stubble Continuous cropping	Clay Loam soil pH 7.1-7.7 Non Saline 3.4% OM	0-15 15-30	21.28 19.04	53.76	>540	>43 38.08
Saskatoon	SW 34-36-6 W3 Moist Dark Brown Soil Zone Previous Crop Canola Spread Stubble Continuous cropping	Clay Loam soil pH 6.9-7.2 Non Saline 4.9% OM	0-15 15-30	20.16 7.84	19.04	>540	34.72 >43
Vanscoy	SE 20-36-6 W3 Moist Dark Brown Soil Zone Previous Crop Wheat Spread Stubble Continuous cropping	Clay soil pH 7.4-8.1 Non Saline 3.3% OM	0-15 15-30	19.04 24.64	56	>540	13.44 13.44
Cabri	SE 23-19-18 W3 Brown Soil Zone Previous Crop Wheat Spread Stubble Non-continuous cropping	Clay soil pH 8.2 Non Saline 2.2% OM	0-15	6.72	7.84	>510	23.52

5.4.2 Agronomic procedures

Wheat was seeded directly into standing canola and cereal stubble. Seeding dates ranged from May 17 to May 25, 2009. Soil moisture during the time of seeding was poor at all locations. Lillian wheat was seeded at 90 kg ha^{-1}.

During the winter of 2008/2009 the seeder was overhauled with new shanks and some changes to the air flow system. The width was also increased between seed rows. Seed was still metered using a Monosem vacuum metering system. Shanks were changed to a Bourgault double-shoot system with side-band tips (Dutch Industries, Pilot Butte, SK). Seed rows were changed to 30 cm spacing. This system was also changed to hydraulic for more uniform seeding depth in the soil. Seed was placed 2.5 cm above and 2.5 cm to the side of the fertilizer. Wheat was seeded 3 cm deep, directly into standing stubble.

Plots were treated as they were in 2008. Seed-placed 0-45-0 was applied at a rate of 24 kg ha^{-1}, glyphosate was sprayed prior to seeding, and in crop weeds were controlled with a foliar application of clodinafop-propargyl for grassy weeds and MCPA, mecoprop-p and dicamba for broadleaf weeds. Desiccation at harvest was performed with diquat. All pesticides were applied at the manufacturer's recommended rates. The weeds that were not controlled chemically were removed manually at various times throughout the growing season.

5.4.3 Inoculation

As was the case in 2008, *G. azotocaptans* and *A. lipoferum* were used in the field trials. It was found over the winter of 2008/2009 that these strains were not stable. The diazotroph species *A. lipoferum* was found to be stable for one week when stored at 4, 15 and 20°C, but at 2 weeks titres declined considerably, especially when stored at 4°C. *Gluconacetobacter azotocaptans* was more stable when stored at 4 and 15°C but not at 20°C, and again titres decreased after one week of storage (Mary Leggett, personal communication). New inoculant was produced every week in 2009, during seeding, to ensure high titre numbers on the seed at inoculation. Strains were produced using the same method as described for the lab experiment (section 3.3.1).

Inoculants were applied at $1.07x10^9$ CFU *A. lipoferum* per gram of wheat seed, and $1.05x10^9$ CFU *G. azotocaptans* per gram of wheat seed. The uninoculated control was treated with the same volume of water as the inoculant strains. The inoculants and seed samples were combined in plastic bags in the field and shaken vigorously until there was even application of inoculant on the seed.

5.5 Experimental design and treatments

The wheat plots were designed in a split-plot with six replicated blocks. The main treatments were 0, 20, 40, 60 and 80 kg N ha^{-1} of granular urea (46-0-0) that was side banded. Sub-plot treatments consisted of uninoculated wheat that was treated with water, and wheat treated with either *G. azotocaptans* or *A. lipoferum*. A detailed list of treatments is presented in Table 5.3. Plots were 10 m in length by 1.5 m and contained 5 wheat seed rows with a guard row consisting of lentils on either side of the plot (Appendix D).

Micro-plots (1 m^2) were established right after seeding within the subplots of the 0, 40, and 80 kg N ha^{-1} treatments, at two locations each year (Langham and Vanguard in 2008, and Langham and Saskatoon in 2009) (Appendix E). The micro-plots were treated with 5 kg ha^{-1} of the stable isotope 10 atom% ^{15}N-urea fertilizer (Appendix F). In the field, 10 mL of ^{15}N solution (containing 1.037 g ^{15}N urea per 10 mL) was added to water in a watering can using a syringe and applied to each of the micro-plots. Another half a can of water was then poured over top of the micro-plots to move the ^{15}N down into the rooting zone.

5.6 Sample collection and analysis

Samples were harvested from sites which contained the ^{15}N micro-plots (0, 40 and 80 kg N ha^{-1} plots) at Feekes Growth Stage 8-9 (flag-leaf) which represents the maximum N fertilizer accumulation in the plant when ^{15}N is used (Griggs et al., 2002), and at Feekes (Large, 1954) growth stage 11, physiological maturity, and stored in cloth bags. In the ^{15}N micro-plots, a 0.5 m by 1 m quadrant of above-ground biomass was harvested at the plant/soil interface using knives at flag leaf and harvest. Samples were dried at 40°C for 7 d. Storage containers with

Table 5.3. Treatments applied to field plots in 2008 and 2009.

Treatment	Product	Fertilizer Source	Formulation	Application
	Main Plot			
1	0 kg ha^{-1}	46-0-0	Granular	Side band
2	20 kg ha^{-1}	46-0-0	Granular	Side band
3	40 kg ha^{-1}	46-0-0	Granular	Side band
4	60 kg ha^{-1}	46-0-0	Granular	Side band
5	80 kg ha^{-1}	46-0-0	Granular	Side band
	Sub Plot			
1	Uninoculated control		Water	On seed
2	*Azospirillum lipoferum*		Liquid	On seed
3	*Gluconacetobacter azotocaptans*		Liquid	On seed

dehumidifiers and fans were used to dry plants. Dry samples were weighed and four stems were randomly picked from each mature sample and split into heads and stems for analysis. Samples were ground using a Wiley mill to pass through a 20 mesh sieve and then pulverized in a rotating ball mill. Ethanol was used to clean the mill between samples after vacuum cleaning to prevent ^{15}N cross contamination. The ^{15}N-labeled plants from the microplots were then analysed for atom% ^{15}N and %N.

A 1 m^2 quadrant of above-ground biomass was harvested from non-enriched plots (20 and 60 kg N ha^{-1} plots) at Feekes Growth Stage 8 and 11 (Large, 1954). These samples were stored in cloth bags, and dried at 40°C for 7 d. Storage containers with dehumidifiers and fans were used to dry plants. Samples from non-enriched plots were ground using a Wiley mill to pass through a 20 mesh sieve, then analyzed at AgVise Laboratories (Northwood, ND, USA) using an Elementar Rapid-N Cube combustion analyzer for %N uptake only. Four stems were randomly picked from each mature sample and split into heads and stems for analysis.

All plots were harvested using a Wintersteiger combine at physiological maturity. Weights and moisture readings were recorded by a Harvest Master® system (Juniper Systems, Logan, UT) at time of harvest. Seed samples were collected from each plot.

Of the four sites planted in 2008, only two were harvested. There were emergence issues at the Saskatoon site and the crop never fully emerged, therefore it was ploughed under in June 2008. The site at Vanguard was one of the two selected for ^{15}N enrichment. Hail destroyed the crop in July 2008 before any sampling could be done. Langham was the second site with ^{15}N enriched micro-plots; plant samples and harvest data were recorded at this site. Loreburn was also harvested. Due to the possible inconsistencies of stability of the inoculant in 2008, the data collected in 2008 was not included.

In 2009, originally four sites were established. However, the Delisle site was the first seeded and had to be taken out of production. Issues had arisen with temperatures not being constant in the storage chambers the *A. lipoferum* and *G. azotocaptans* had been stored in. A fifth site was then added at Cabri, SK. The Vanscoy site did not emerge and was removed from production in June, 2009. Saskatoon and Langham micro-plots (0, 40 and 80 kg N ha^{-1}

treatments) were sampled and analysed for for dry matter, atom% ^{15}N, %N, and grain yield. At these two sites, the non-enriched plots (20 and 60 kg N ha^{-1} treatments) were sampled for dry matter, N uptake and grain yield. The Cabri site was harvested for grain yield.

5.7 Nitrogen analysis

Nitrogen fixation was estimated using the ^{15}N isotope dilution method. Traditionally, the method compares the uptake of ^{15}N-enriched fertilizer between an N_2-fixing plant and a non-N_2-fixing plant. The fixing plant has three sources of N available to it; soil N, the enriched fertilizer and non-enriched N from fixation, whereas, the non-fixing plant has only two sources of N, soil N and ^{15}N-enriched fertilizer. When actively fixing, the N in the fixing plant biomass is diluted by the non-enriched atmospheric N compared to the non-fixing reference plant. In this study the technique is applied to the diazotrophs. If the diazotroph is actively fixing N and this N is taken up by the wheat, the ^{15}N signal will be lower (more dilute) than wheat that is only taking up N from the applied fertilizer.

Nitrogen content in plant tissues was determined using a Leco 2000 analyzer; and atom %^{15}N was determined by mass spectrometry using a Costech ECS4010 elemental analyzer, coupled to a Delta V mass spectrometer with Conflo IV interface.

The percentage of N derived from the atmosphere (%Ndfa) was calculated by the following equation:

$$\%\text{Ndfa} = \left(1 - \frac{\text{atom}\,\%^{15}\text{N excess}_{(F)}}{\text{atom}\,\%^{15}\text{N excess}_{(NF)}}\right) * 100 \qquad [5.1]$$

Where %Ndfa is the percentage of N derived from the atmosphere; atom% ^{15}N excess is the enrichment of the fixing (F) and non-fixing (NF) crops with the natural abundance ^{15}N of 0.3663 subtracted, respectively (Hardarson and Danso, 1990; Danso et al., 1993; Boddey et al., 1995; IAEA, 2001). The fixing crop is wheat that was inoculated with either *G. azotocaptans* or *A. lipoferum*, and the non-fixing crop is wheat that was uninoculated.

The amount of N uptake (or N yield) was calculated by using a harvest index of four plants from the ^{15}N microplot at maturity. The whole sample was weighed to give a total

biomass, and then four plants from the sample were split into heads and stems and weighed. The harvest index was calculated by dividing the weight of seed or straw of the four plants by the total weight of the four plants. It was assumed that the harvest index was representative of the total sample. Then the harvest index (seed or stem) was multiplied by the total biomass of the sample to give the weight of heads or stems in the whole sample. Nitrogen uptake was then calculated by taking the %N value from analysis and multiplying it by the weights of the plant parts.

The amount of fixed N was calculated using Equation 5.2. Where, N yield is the total N content in plant parts.

$$N_2 \text{ fixed } (\text{kg ha}^{-1}) = \frac{\%\text{Ndfa}}{100} \text{ x N yield } (\text{kg ha}^{-1}) \qquad [5.2]$$

5.8 Statistical analysis

Results were analyzed by location and as total combined data. There were no differences between the analyses; therefore data was reported as combined location analysis. Combined location data acquired at the flag leaf and harvest stages were analyzed using the standard least squares procedure in SAS (JMP Version 8.0.2, SAS Institute Inc. Cary, NC, USA). If significance (P<0.05) was found for any effects, a Tukey's t-test was performed. The treatments were categorized by N applications and seed treatments at each site.

5.9 Results

5.9.1. Dry matter

Statistical analyses carried out for the two locations for the amount of dry matter accumulation of above-ground plant material showed that fertilization was significant (P<0.05) at both harvest times, as well as for mature heads and stems (Table 5.4). For all harvest times and plant parts, as fertilizer levels increased, so did the amount of dry matter (Figure 5.1a).

Table 5.4 Analysis of variance of two combined locations (Loc), Langham and Saskatoon, for above-ground dry matter of wheat inoculated with three seed treatments (Trt); at five fertilizer levels (Fert); at two harvest times; over six replicated blocks (Rep). Plants at maturity were separated into mature total biomass, heads and stems.

Harvest		**Random Effects**			**Fixed Effects**			
Biomass	**Components at Maturity**	**Random Effect**	**Var Ratio**	**Std Error**	**Source Var**	**Deg Freedom**	**F Ratio**	**Prob>F**
Flag-leaf		Rep(Loc)	0.41	37.09	Loc	1	0.7336	0.414
		RepxFert(Loc)	0.56	28.67	Fert	4	45.7938	<0.0001*
		Residual		18.95	Trt	2	0.3461	0.7083
					Fert x Trt	8	1.1097	0.3634
					Loc x Fert	4	0.2457	0.9104
					Loc x Trt	2	1.9251	0.1513
Maturity	Total Biomass	Rep(Loc)	0.33	231.43	Loc	1	1.5290	0.2476
		RepxFert(Loc)	1.05	258.16	Fert	4	41.5870	<0.0001*
		Residual		112.15	Trt	2	0.4179	0.6596
					Fert x Trt	8	0.4666	0.8769
					Loc x Fert	4	2.6231	0.0507
					Loc x Trt	2	0.5750	0.5646
	Heads	Rep(Loc)	0.30	163.5	Loc	1	12.2823	0.0067*
		RepxFert(Loc)	1.27	205.17	Fert	4	27.8126	<0.0001*
		Residual		76.72	Trt	2	0.0244	0.9757
					Fert x Trt	8	0.4077	0.9137
					Loc x Fert	4	2.9188	0.0345*
					Loc x Trt	2	0.0230	0.9773
	Stems	Rep(Loc)	0.21	25.08	Loc	1	26.4049	0.0006*
		RepxFert(Loc)	0.27	23.68	Fert	4	35.0048	<0.0001*
		Residual		22.33	Trt	2	2.7841	0.0667
					Fert x Trt	8	0.6028	0.7735
					Loc x Fert	4	1.5416	0.2110
					Loc x Trt	2	2.8692	0.0615

*significant interaction ($P < 0.05$)

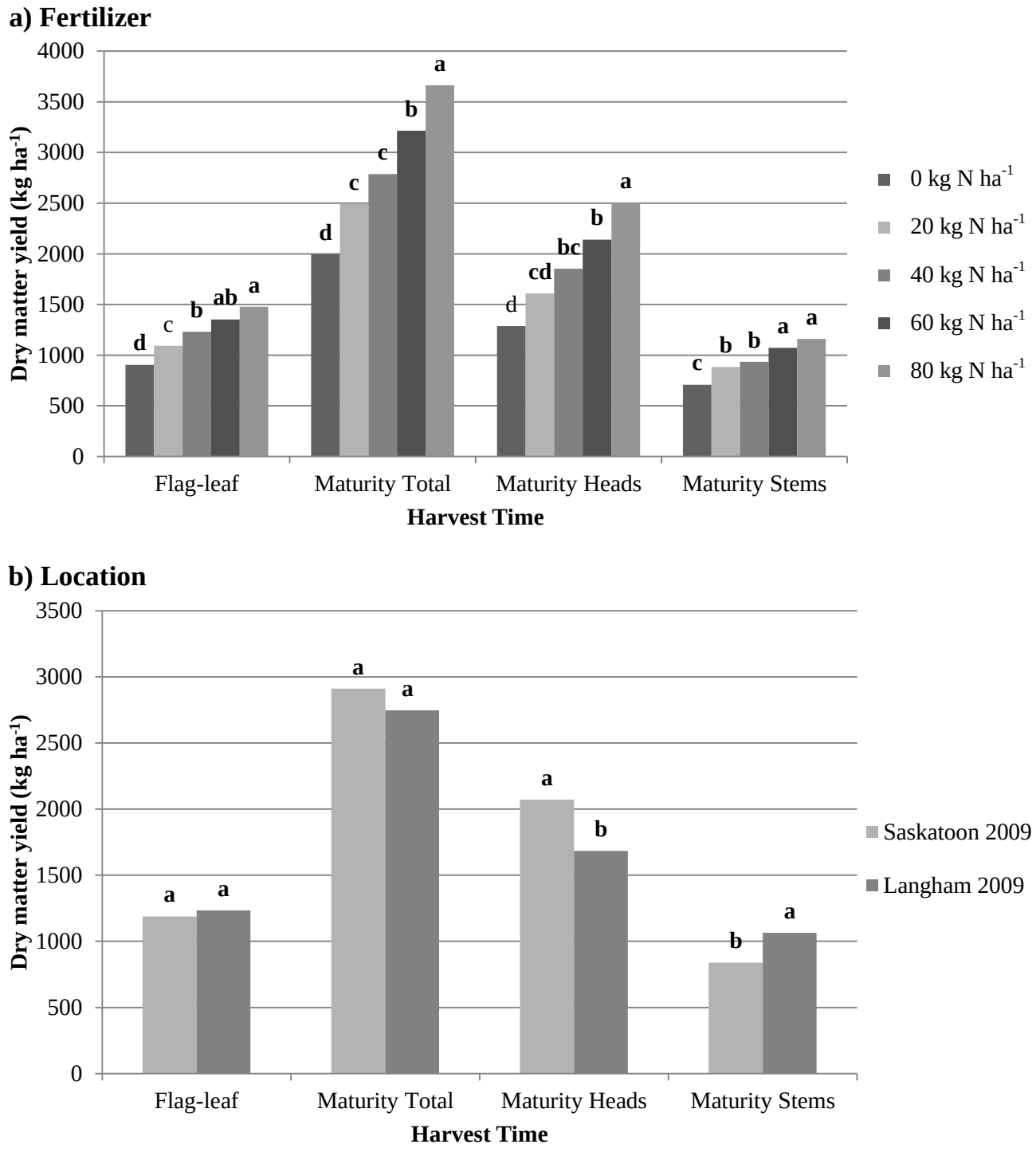


Figure 5.1 The amount of dry matter accumulation of wheat based on the effects from a) fertilizer levels and b) location. There were no significant effects to report from the three seed treatments on dry matter. Within each harvest time or plant part, bars with the same letter are on significantly different according to a Tukey's t-test (P<0.05).

Location was significant (P<0.05), for dry matter yield of heads and stems. The Saskatoon site had significantly (P<0.05) more accumulation of dry matter for heads compared to the Langham site (2072 and 1683 kg ha^{-1} respectively) (Figure 5.1b). However, the Langham site had significantly (P<0.05) more dry matter accumulated than the Saskatoon site when stems were analyzed (1064 and 839 kg ha^{-1} respectively).

5.9.2 Nitrogen derived from the atmosphere

The %Ndfa in wheat plants was calculated using equation 5.1. Combined analysis of the Saskatoon and Langham sites showed fertilizer had a significant effect (P<0.05) on %Ndfa in inoculated wheat (total biomass material) as well as heads and stems analyzed separately (Table 5.5).

With the exception of plants harvested at the flag-leaf stage, as fertilizer levels increased, so did the %Ndfa in wheat plants and plant parts (Figure 5.2). With the addition of 80 kg N ha^{-1}, 3.1% N was fixed at flag-leaf, 10.5% at maturity, 4.8% in heads, and 5.8% in stems. However, when the analysis was done for the inoculant treatments *A. lipoferum* and *G. azotocaptans* separately, no significant (P<0.05) difference in %Ndfa occurred for the different diazotroph treatments (data not shown).

5.9.3 Nitrogen uptake

Analysis of the two locations combined showed that fertilizer had a significant (P<0.05) effect on N uptake in wheat plants at both harvest times as well as for heads and stems analyzed separately (Table 5.6). Similar to dry matter accumulation, location was also significant (P<0.05) for the analysis of N uptake in heads and stems separately.

As fertilizer levels increased, so did the amount of N taken up into wheat plants (Figure 5.3a). Plots with no fertilizer additions had significantly (P<0.05) lower amounts of N uptake than most other fertilizer treatments at all harvest stages. This pattern was seen at both harvest times as well as with mature heads and stems. The highest amount of N uptake in plants was

Table 5.5 Analysis of variance of two combined locations (Loc), Langham and Saskatoon, for percent nitrogen derived from the atmosphere of wheat inoculated with three seed treatments (Trt); at five fertilizer levels (Fert); at two harvest times; over six replicated blocks (Rep). Plants at maturity were separated into mature total biomass, heads and stems.

Harvest		Random Effects			Fixed Effects			
Biomass	**Components at Maturity**	**Random Effect**	**Var Ratio**	**Std Error**	**Source Var**	**Deg Freedom**	**F Ratio**	**Prob>F**
Flag-leaf		Rep(Loc)	3.88	2.79	Loc	1	0.0162	0.9015
		RepxFert(Loc)	6.42	2.14	Trt	1	0.6636	0.4219
		Residual		0.24	Fert	2	1.2403	0.3129
					Fert x Trt	2	0.6314	0.5390
					Loc x Fert	2	0.4624	0.6370
					Loc x Trt	1	0.0004	0.9847
Mature	Total Biomass	Rep(Loc)	-0.18	2.81	Loc	1	2.6193	0.1400
		RepxFert(Loc)	3.16	5.42	Trt	1	0.0005	0.9831
		Residual		1.15	Fert	2	12.6817	0.0004*
					Fert x Trt	2	0.3828	0.6854
					Loc x Fert	2	0.1373	0.8726
					Loc x Trt	1	1.3469	0.2553
	Heads	Rep(Loc)	1.28	1.52	Loc	1	0.9974	0.3440
		RepxFert(Loc)	1.82	1.19	Trt	1	0.8788	0.3563
		Residual			Fert	2	10.009	0.0012*
					Fert x Trt	2	0.3915	0.6795
					Loc x Fert	2	0.1862	0.8317
					Loc x Trt	1	0.0774	0.7828
	Stems	Rep(Loc)	-0.61	0.84	Loc	1	4.7434	0.0574
		RepxFert(Loc)	2.64	2.20	Trt	1	0.8469	0.3650
		Residual		0.54	Fert	2	10.9529	0.0008*
					Fert x Trt	2	1.1814	0.3212
					Loc x Fert	2	0.1132	0.8936
					Loc x Trt	1	1.6835	0.2047

*significant interaction ($P<0.05$)

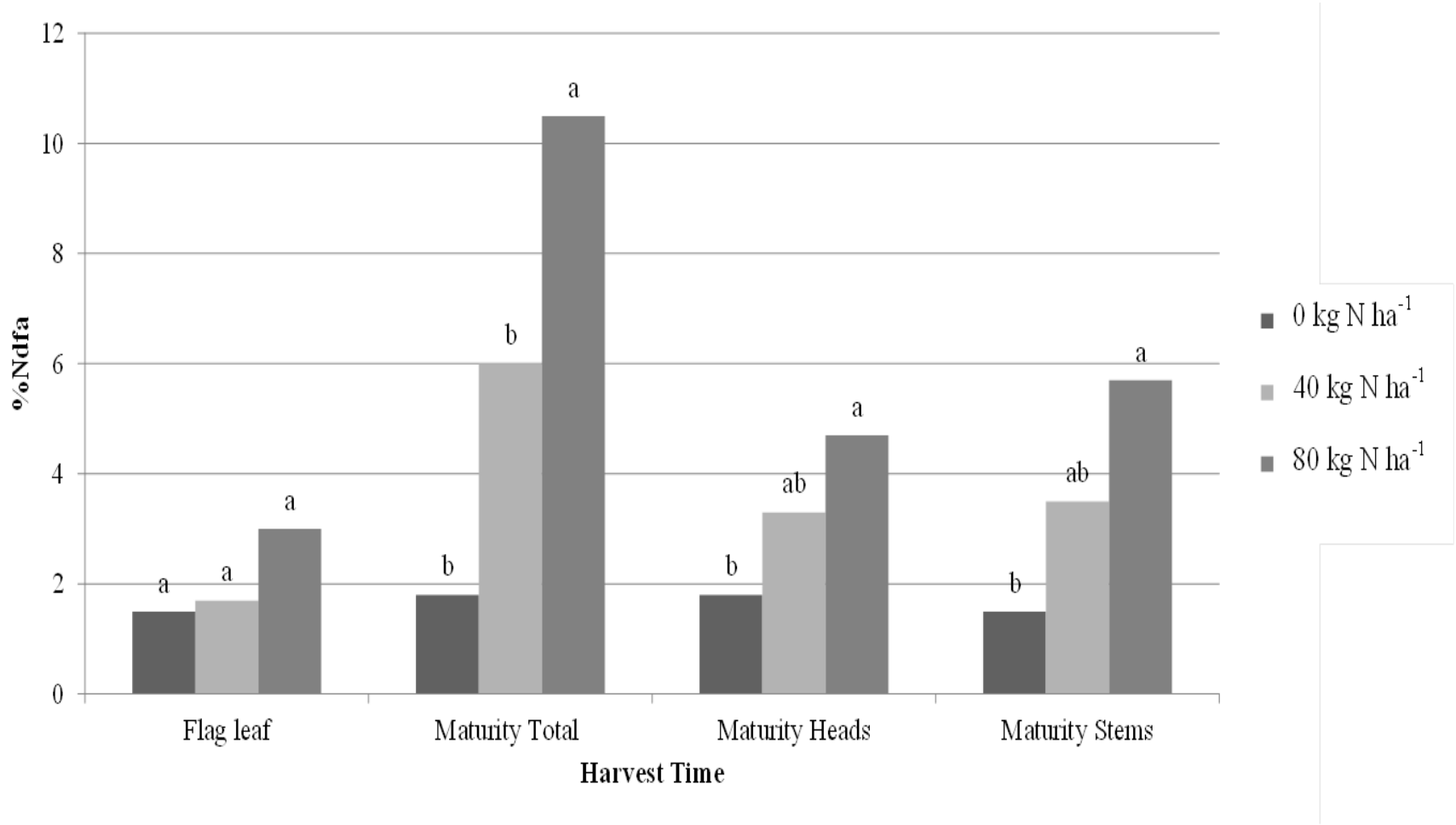


Figure 5.2. The influence of fertilizer rate on the percent nitrogen derived from atmosphere in wheat at two harvest times. Within each harvest time or plant part, bars with the same letter are not significantly different according to a Tukey's t-test ($P<0.05$)

Table 5.6 Analysis of two combined locations (Loc) for N uptake in wheat inoculated with three seed treatments (Trt); at five fertilizer levels (Fert); at two harvest times; over six replicated blocks (Rep). Plants at maturity were separated into mature total material, heads and stems.

Harvest		Random Effects			Fixed Effects			
Biomass	**Components at Maturity**	**Random Effect**	**Var Ratio**	**Std Error**	**Source Var**	**Deg Freedom**	**F Ratio**	**Prob>F**
Flag-leaf		Rep(Loc)	0.05	1.18	Loc	1	3.1484	0.1097
		RepxFert(Loc)	0.76	2.26	Fert	4	78.8968	<0.0001*
		Residual		1.22	Trt	2	0.0477	0.9535
					Fert x Trt	8	0.9022	0.5179
					Loc x Fert	4	0.3020	0.8747
					Loc x Trt	2	0.2471	0.7815
Maturity	Total Biomass	Rep(Loc)	0.01	4.07	Loc	1	0.5677	0.4704
		RepxFert(Loc)	1.43	8.86	Fert	4	27.3132	<0.0001*
		Residual		3.02	Trt	2	1.0963	0.3382
					Fert x Trt	8	0.8170	0.5894
					Loc x Fert	4	0.5608	0.6926
					Loc x Trt	2	0.9874	0.3762
	Heads	Rep(Loc)	-0.03	3.17	Loc	1	5.6306	0.0417*
		RepxFert(Loc)	1.36	7.76	Fert	4	21.7503	<0.0001*
		Residual		2.76	Trt	2	0.7224	0.4882
					Fert x Trt	8	0.7521	0.6455
					Loc x Fert	4	0.9364	0.4540
					Loc x Trt	2	0.9581	0.3872
	Stems	Rep(Loc)	0.15	0.26	Loc	1	25.0070	0.0007*
		RepxFert(Loc)	1.44	0.44	Fert	4	16.8947	<0.0001*
		Residual		0.15	Trt	2	1.2826	0.2819
					Fert x Trt	8	1.5342	0.1552
					Loc x Fert	4	1.4350	0.2424
					Loc x Trt	2	2.7282	0.0703

*significant interaction ($P < 0.05$)

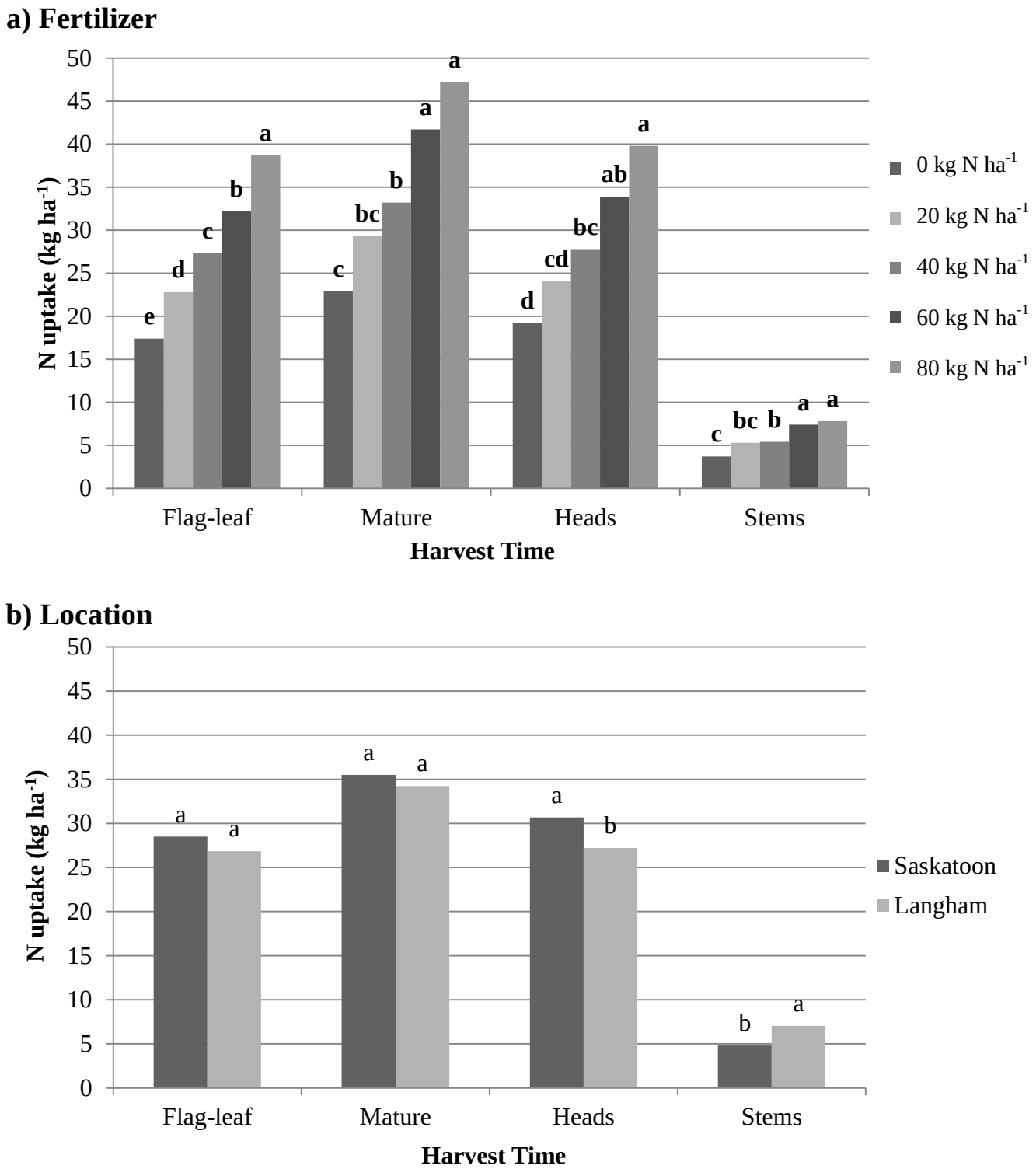


Figure 5.3 The influence of a) fertilizer rate, and b) location on the amount of nitrogen uptake in inoculated wheat at two harvest times. Within harvest times or plant parts, bars with the same letter are not significantly different according to Tukey's t-test (P<0.05).

from plots fertilized with 80 kg N ha^{-1}, with 38.7 kg ha^{-1} taken up at flag-leaf, 47.2 kg ha^{-1} at maturity, 39.8 kg ha^{-1} in heads, and 7.8 kg ha^{-1} in stems.

No differences were found in N uptake between Langham and Saskatoon at flag-leaf or mature harvest (Figure 5.3b). However, analysis of heads showed that wheat at the Saskatoon site had significantly (P<0.05) more N than wheat at the Langham site (30.7 and 27.2 kg ha^{-1} respectively). Stems showed the opposite effect. There was significantly (P<0.05) more N uptake in wheat stems at the Langham site compared to the Saskatoon site (7.0 and 4.8 kg ha^{-1} respectively).

5.9.4 Amount of nitrogen fixed

Fertilizer had a significant (P<0.05) effect the on amount of N_2-fixed at both harvest times as well as for mature heads and stems (Table 5.7). Fertilization with 80 kg N ha^{-1} resulted in significantly (P<0.05) more N_2-fixed in wheat plants compared to the other two fertilizer levels (Figure 5.4). Plots fertilized with 40 kg N ha^{-1} resulted in more N_2-fixed in wheat plants than plots with no fertilizer additions at mature harvest, and for heads.

5.9.5 Yield

Three sites were harvested mechanically, Saskatoon, Langham and Cabri. The yields for the three locations were combined and analyzed (Table 5.8). Location and fertilizer level had a significant (P<0.05) effect on yield.

Wheat grown with the different fertilizer levels for the three locations combined showed the same pattern that was seen with dry matter and N uptake (Figure 5.5a). The lowest grain yields occurred on plots with no fertilizer added (1646 kg ha^{-1}). As the fertilizer levels increased, so did the yields, each significantly (P<0.05) higher than the last. The maximum yields were recorded for plots with fertilizer additions of 80 kg N ha^{-1} (2314 kg ha^{-1}). The Cabri 2009 location had significantly (P<0.05) lesser yield than Langham and Saskatoon locations (1802, 1880, and 2386 kg ha^{-1}, respectively) (Figure 5.5b). Yields at the Saskatoon site were significantly (P<0.05) higher than the other two sites.

Table 5.7 Analysis of variance of two combined locations (Loc) for the amount of atmospheric fixed N in wheat inoculated with three seed treatments (Trt); at three fertilizer levels (Fert); at two harvest times; over six replicated blocks (Rep). Plants at maturity were separated into mature total material, heads and stems.

Harvest		Random Effects			Fixed Effects			
Biomass	**Components at Maturity**	**Random Effect**	**Var Ratio**	**Std Error**	**Source Var**	**Deg Freedom**	**F Ratio**	**Prob>F**
Flag-leaf		Rep(Loc)	0.21	0.09	Loc	1	0.0031	0.9571
		RepxFert(Loc)	6.79	0.15	Fert	2	6.2526	0.0087*
		Residual		0.02	Trt	1	0.9090	0.3483
					Fert x Trt	2	0.1036	0.9020
					Loc x Fert	2	0.0040	0.9960
					Loc x Trt	1	0.3567	0.5550
Maturity	Total Biomass	Rep(Loc)	-0.04	0.25	Loc	1	2.0413	0.1868
		RepxFert(Loc)	1.32	0.46	Fert	2	38.4166	<0.0001*
		Residual		0.20	Trt	1	0.0937	0.7617
					Fert x Trt	2	0.3819	0.6859
					Loc x Fert	2	0.0423	0.9587
					Loc x Trt	1	2.4914	0.1253
	Heads	Rep(Loc)	0.86	0.08	Loc	1	2.3696	0.1581
		RepxFert(Loc)	0.90	0.06	Fert	2	37.9935	<0.0001*
		Residual		0.03	Trt	1	0.3903	0.5370
		total			Fert x Trt	2	0.3249	0.7252
					Loc x Fert	2	0.4037	0.6738
					Loc x Trt	1	1.6457	0.2097
	Stems	Rep(Loc)	-0.22	0.002	Loc	1	1.7601	0.2173
		RepxFert(Loc)	0.92	0.004	Fert	2	31.7216	<0.0001*
		Residual		0.002	Trt	1	0.4384	0.5131
		total			Fert x Trt	2	1.2969	0.2888
					Loc x Fert	2	0.5397	0.5921
					Loc x Trt	1	1.7397	0.1975

*significant interaction ($P < 0.05$)

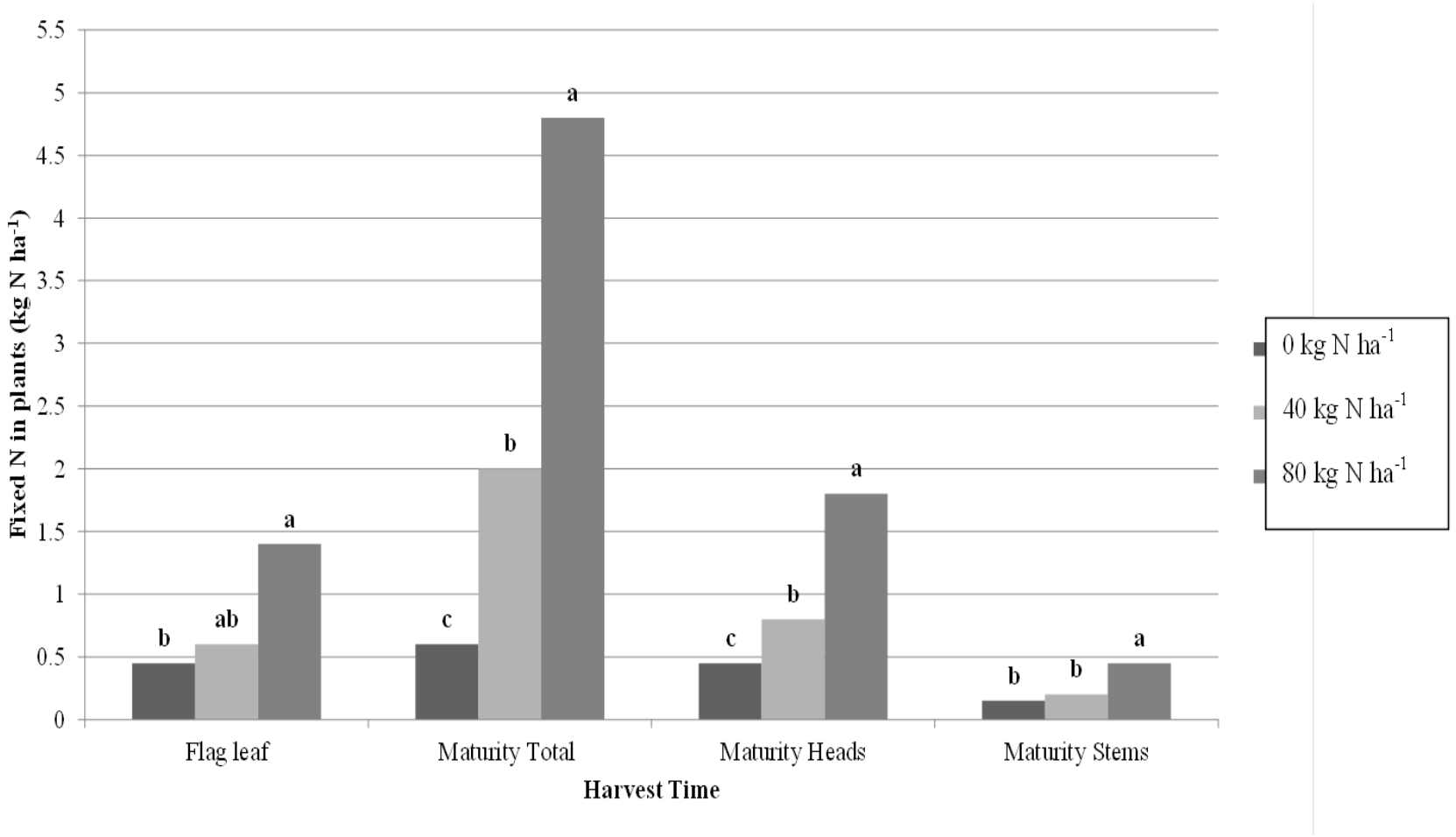


Figure 5.4. The influence of fertilizer rate on the amount of atmospheric fixed nitrogen accumulated in wheat inoculated with *A. lipoferum* and *G. azotocaptans* at two harvest times. Within a harvest time or plant part, bars with the same letter are not significantly different according to Tukey's t-test ($P<0.05$).

Table 5.8 Analysis of variance of three combined locations (Loc) of harvest yields of wheat inoculated with three seed treatments (Trt); at five fertilizer levels (Fert); over six replicated blocks (Rep).

Random Effects			**Fixed Effects**			
Random Effect	**Var Ratio**	**Std Error**	**Source Var**	**Deg Freedom**	**F Ratio**	**Prob>F**
Rep(Loc)	0.003	17.23	Loc	2	13116.10	<0.0001*
RepxFert(Loc)	-0.32	298.50	Fert	4	11952.18	<0.0001*
Residual		893.43	Trt	2	1.0406	0.3557
Total			Fert x Trt	8	5.4387	<0.0001*
			Loc x Fert	8	3.9045	<0.0001*
			Loc x Trt	4	9.6680	<0.0001*

*significant interaction ($P < 0.05$)

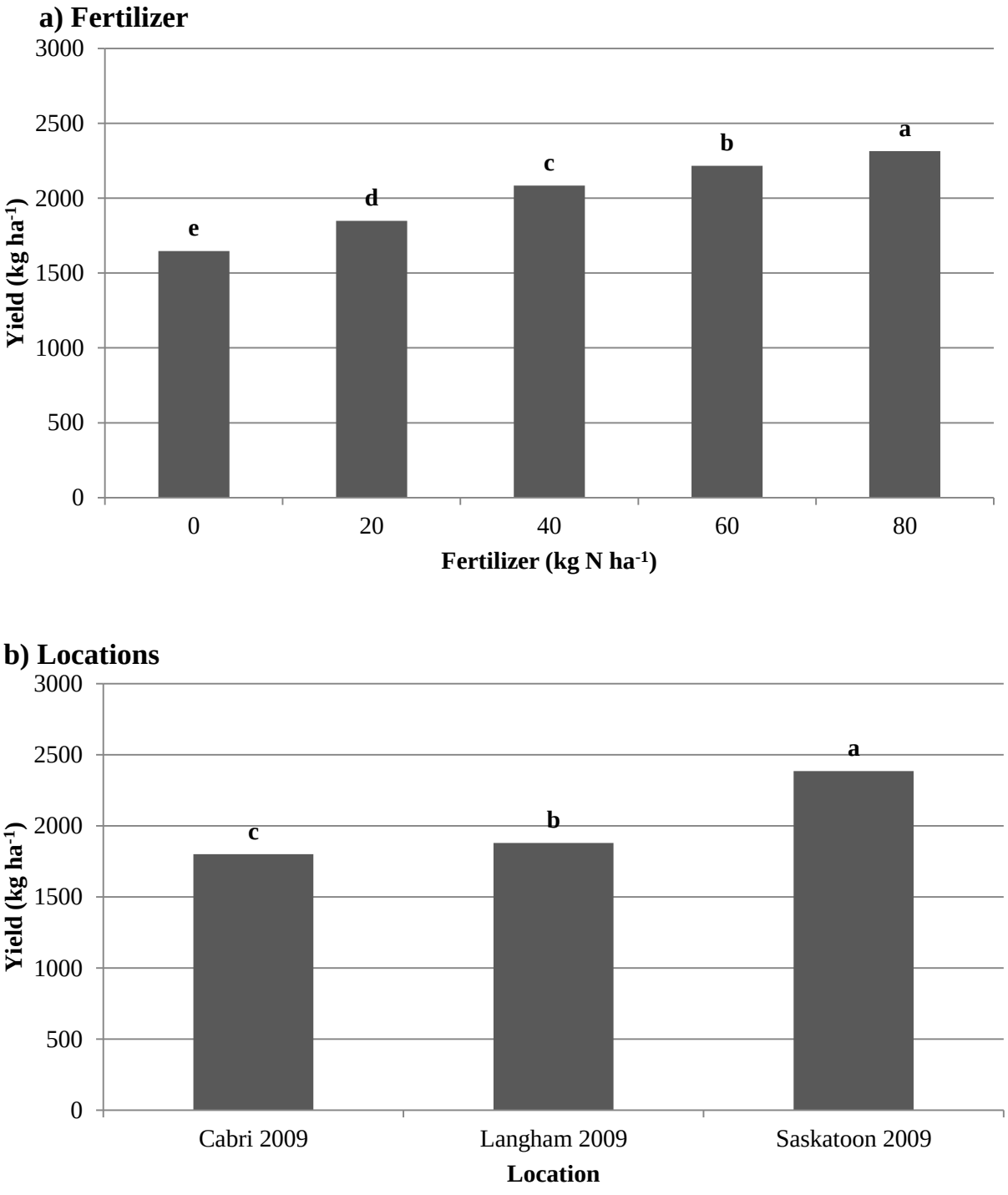


Figure 5.5 The influence of a) fertilizer rate and b) location on grain yields of inoculated wheat at three sites in Saskatchewan. Bars with the same letter are not significantly different according to Tukey's t-test (P<0.05).

5.10 Discussion

Plant growth promoting bacteria (PGPB) belong to genera including *Azospirillum* and *Gluconacetobacter*, among many others. They are capable of promoting plant growth through many different mechanisms including biological N_2-fixation (BNF), phytohormone production, P solubilisation, siderophore production, and root growth promotion (Dobbelaere and Okon, 2003; El Zemrany et al., 2006; Pedraza, 2008). This study examined *A. lipoferum* and *G. azotocaptans* for their effect on wheat plant growth and N uptake in field trials.

Inoculant survival is a prerequisite for the success of beneficial effects on the plant. Survival of inoculated *A. lipoferum* and *G. azotocaptans* will vary according to storage, soil, climatic and plant conditions (Okon and Kapulnik, 1986; Bashan et al., 1995). The multitude of possible interacting factors that might impact the effect of diazotroph inoculation on crop productivity explains low uniformity of results observed in many field studies (Harris et al., 1989; Anyia et al., 2004; Diaz-Zorita and Fernandez-Canigia, 2008). In this study, questions were raised after the 2008 field season as to the viability of both inoculants in storage before seeding. It was found that *A. lipoferum* had a rapid decline in CFU after 2 weeks in storage, furthermore *G. azotocaptans* rapidly declined after only 1 wk. Therefore, results from 2008 were compromised since inoculant was prepared for the field trials in late April and early May and was stored until sites were seeded in mid to late May. Any results from the 2008 field season were removed from the data set.

No significant effects on wheat dry matter production and N uptake from inoculation alone were observed in this study at any harvest time. However, some N_2-fixation was occurring based on the calculation of %Ndfa. At the flag leaf harvest the %Ndfa ranged from 1.5 to 3.1% in wheat plants. These values increased as the year progressed and fixation from the diazotroph species continued. At maturity %Ndfa ranged from 1.9% with no fertilization to 10.5% with 80 kg N ha^{-1}. There were no significant differences observed when %Ndfa was analysed to compare *A. lipoferum* or *G. azotocaptans* inoculated wheat.

Not surprisingly, analysis of dry matter, N uptake, and yield showed that fertilization was significant in this study. A common pattern emerged throughout the research. As fertilizer levels increased, so did the amounts or degree of the tested variable, be it dry matter, or N

accumulation. This was an expected outcome because of the importance of N in plant growth, as a structure of protein as well as being found in chlorophyll (Novoa and Loomis, 1981).

Contrary to what is seen with rhizobia inoculation, fertilizer did not inhibit N_2-fixation in this experiment. The %Ndfa in wheat plants inoculated with *A. lipoferum* and *G. azotocaptans* correlating to increases in fertilization was similar to the pot experiment in Chapter 4 where 12.2 and 24.5 µg N g^{-1} (equivalent to 40 and 80kg N ha^{-1} respectively) had the highest amounts of %Ndfa in wheat plants. Rennie and Thomas (1987) found similar results in wheat despite high soil N (52.5 kg to a 60 cm depth). In that study, amounts of N_2-fixed were large enough to be agronomically significant both in terms of average response to inoculation (17.9 kg ha^{-1} in 1984 and 6.7 kg ha^{-1} in 1985) and in terms of the peak response (52.4 kg ha^{-1} in 1984 and 31.3 kg ha^{-1} in 1985).

High fertilizer rates result in larger plants which are assumed to have larger root systems. These larger root systems might promote larger quantities of carbon (C) through root exudations and root turnover, providing a C source for *A. lipoferum* and *G. azotocaptans.* Larger populations of the diazotrophs would, in turn, fix more N. In another study, after 70 h insignificant amounts of newly fixed N were transferred from an ammonia-excreting strain of *A. brasilense* to the shoot tissue of wheat (Wood et al., 2001). However, when malate was added to the co-culture, the ^{15}N enrichment of the shoot tissue increased 48-fold indicating that 20% of shoot N had been derived from N_2-fixation. Thus, the inability of the host plant to release C in the rhizosphere can be a significant constraint in the development of associative N_2-fixing systems.

Pulse crop and canola residues usually contain higher concentrations of N and P, and therefore return more of those nutrients to the soil than cereal residues (Strong et al., 1986; Armstrong et al., 1994). Residue decomposition and subsequent release of N depends on the chemical composition of crop residues. Easily decomposable materials may be decomposed and the nutrients lost soon after, whereas more resistant materials can persist for much longer (Paul and Clark, 1996). Differences in decomposition reflects the differences in chemical composition of the crop residues, and it is the N concentration in the plant material, or more specifically, the ratio of C:N that is the best predictor of N mineralization rates (Janzen and Kucey, 1988; Walley, 2005). This could be a reason that the Saskatoon site that was grown on canola stubble had more

fixation occurring, more N uptake in the wheat plants, and higher harvest grain yields. Ratios of C:N greater than 20:1 to 30:1 result in rapid immobilization of N from the inorganic N pool. Wheat residues (C:N ratio of 70:1 and greater) typically cause N to be temporarily immobilized by microbes (Soon and Arshad, 2002; Walley, 2005).

Positive results from this research must be due to the activity of *G. azotocaptans* or *A. lipoferum* inoculated on wheat seed. There are, however, three possible mechanisms of such activity: N_2-fixation, production of plant growth substances, and interaction with nitrate assimilation of the plant (Patriquin et al., 1983; Mertens and Hess, 1984). Additionally, the possibility of moderately indirect bacterial effects on wheat growth and development cannot be completely excluded.

In conclusion, *A. lipoferum* and *G. azotocaptans* were able to fix atmospheric N and that became available for uptake by wheat plants. Based on results from %Ndfa, it can be assumed that the diazotroph species were able to colonize the rhizosphere and survive in the soil and plant environment. Nonetheless, there were no significant effects on dry matter production, N uptake, or yield due to inoculation with either *A. lipoferum* or *G. azotocaptans*. The multitude of possible interacting factors that could impact inoculation of crop productivity is a major concern, therefore continued research under multiple environmental and crop management conditions should continue to give a more comprehensive understanding of the benefits diazotrophs can provide to a crop.

6. CONCLUSION

The ambitious goal of achieving nitrogen (N_2)-fixation by cereals is controversial. However, despite the biological obstacles, there are strong and obvious grounds for making an attempt to achieve it, based on environmental and economic considerations (Kennedy et al., 1997). Nitrogen from biological N_2-fixation (BNF) can be used directly by the plant, and therefore is less susceptible to volatilization, denitrification and leaching. Cocking (2002) has called for concerted action to encourage biofertilizer production associated with BNF to become a more significant feature of world agriculture. This would help to overcome chronic problems such as low farm productivity and poor returns on labour referred to by Reeves et al. (2002). It is also in keeping with the trend towards a more organically and sustainable based agriculture.

The endophytic colonization of sugarcane by free-living microorganisms presents a monocot/diazotroph association. If one grass can arrange to harbour an endophyte and benefit from the association, is it possible that other grasses can also? In western Canada it was meaningful to think of other cereals, like wheat, being encouraged to act as hosts to the diazotrophs, specifically *Azospirillum lipoferum* and *Gluconacetobacter azotocaptans*, and possess endophytic N_2-fixation. Inoculation of wheat with *A. lipoferum* has been widely studied (Mertens and Hess, 1984; Creus et al., 1996; Iniguez et al., 2004), yet inoculation of wheat with *G. azotocaptans* is relatively new.

Nitrogen fixation varies with the organism, plant, environmental conditions, and nutritional status of the soil. However, the commercial significance of associative N_2-fixation in many plants, and the study of biological N_2-fixing bacteria, remains important and necessary. Further experiments will need to be carried out to answer questions about the bacterial establishment, colonization process, diazotroph-plant interactions, BNF, etc., not only with *A. lipoferum* but also with *G. azotocaptans* and other N_2-fixing acetic acid bacteria.

6.1 Inoculation

Microbial inoculants have long been incorporated into field practices, with satisfactory results, especially for rhizobia (Smith et al., 1981; Somasegaran, 1985). There have been many studies on inoculation with N_2-fixing bacteria of non-legumes, with reported above-ground

increases in total plant dry weight, plant N in shoot and grain, numbers of tillers, ears, spikes and grains per spike, and increases in root length and volume (Bashan and Levanony, 1990). The most successful inoculation responses have been in pot trials under controlled conditions (e.g., Negi et al., 1987; Aly et al., 1999; Yanni and Abd El-Fattah, 1999; Alam et al., 2001). Inoculation experiments in the field have been less consistent. For example, Baldani et al. (1987) found increases in grain yield in wheat of up to 31% following inoculation with *Azospirillum* spp., but due to variability in the trials, there were no statistical differences between inoculated plants and untreated controls. Das and Saha (2003) observed increases of up to 20% in grain yield in rice in response to inoculation with *Azotobacter* spp. and *Azospirillum* spp., but these increases were less than those recorded with optimum N fertilizer application.

In this study, N derived from the atmosphere (%Ndfa) was consistently found in the diazotroph treated plants, however no significant ($P<0.05$) differences were found between *A. lipoferum* or *G. azotocaptans*. These results were consistent for both the growth chamber experiment and the field trial. However, even though there was an increase in atmospheric N in the plants there was no significant ($P<0.05$) difference between the grain yields for any inoculant treatment.

Inoculum formulation and application technology are likely to be crucial for inoculant survival and success (Bashan, 1998). Fages (1992) suggested that micro-granulated inoculum similar to that used for rhizobia is likely to be the most successful. However, in this study liquid inoculum was used as a carrier based on easier application on seeds, and ensuring a specific bacterial load on the seed at the time of inoculation.

Desiccation of liquid inoculants can occur quickly, however this can be counteracted with additives such as polymers. The liquid formulation for *A. lipoferum* and *G. azotocaptans* did not include polymers in this study. The C source and starch content of a liquid formulation is also very specific for each diazotroph species (Ferreira et al., 2003; Vendan and Thangajaru, 2007). Ferreira et al. (2010) showed that there were intrinsic differences among tested microorganisms concerning the best carrier medium in terms of cell survival and viability when carriers of different compositions were compared.

6.2 Competition and compatibility

One difference between rhizobia and non-symbiotic inoculants is that rhizobia are protected once they have infected the legume root, whereas non-symbiotic N-fixing bacteria destined for the rhizosphere or soil must continue to compete successfully with indigenous microflora. Diazotrophs, such as *A. lipoferum* or *G. azotocaptans*, which associate with a host plant in an endophytic relationship, are more likely to be successful inoculants because they can escape competition from indigenous microflora. There have been many studies conducted on soil microorganisms and the interactions with diazotrophs (Schimel, 1995; Buckley and Schmidt, 2003; Bashan et al., 2004; Munoz-Rojas et al., 2005; Hsu and Buckley, 2009). However, none of these previous studies have used *A. lipoferum* or *G. azotocaptans* as inoculants.

One of the difficulties of inoculating seeds or soils with bacteria is that the inoculants generally decline rapidly due to competition with the native microflora (Schank and Smith, 1984; Rao et al., 1987; Bashan et al., 2004; Munoz-Rojas et al., 2005; Hsu and Buckley, 2009). However, there is not much information regarding competition on seeds, or how *A. lipoferum* or *G. azotocaptans* compete with other microorganisms. In the lab study, results showed that competition was not an influencing factor influencing declines in CFU on wheat seed. Both *A. lipoferum* and *G. azotocaptans* were able to compete with native microorganisms based on CFU numbers recovered off the seed compared to recovery of CFU off of sterile seed. However, this does not mean that competition may still be occurring.

In the soil, inoculants compete for available nutrients or become food for indigenous micro- and macro-fauna. Hence the ultimate test, for even the most effective beneficial organisms, is the ability to survive and colonise plant roots in the presence of much larger populations of indigenous microorganisms (Bashan and Levanony, 1990). This study did not directly look at soil competition in the growth chamber and field experiments. Enumeration of native microorganisms on the seed coat and in the soil could have been beneficial before the experiments began. This would have given an idea as to the amount of competition occurring for the introduced diazotroph species.

The lab experiment showed that both *A. lipoferum* and *G. azotocaptans* were able to survive on unsterilized seed but it was unclear if they would survive in the soil. For the growth chamber and field experiments, it was assumed that if inoculated wheat showed uptake of fixed N, then *A. lipoferum* or *G. azotocaptans* were able to compete in the soil rhizosphere and colonize along the roots of wheat plants. Based on results in this current study, both *A. lipoferum* and *G. azotocaptans* were able to compete and survive against native microorganisms, either on the seed or in the soil.

Seed applied fungicides are used in western Canada to reduce the establishment of seed- and soil-borne disease organisms (Ahmad and Khan, 2010). However, there can be instances where the fungicide becomes toxic to bacteria applied as inoculants. During the lab experiment, it was found that *G. azotocaptans* had the lowest recovery off of wheat treated with Dividend XL® RTA®, however no significant ($P<0.05$) differences were observed during slope comparisons with the other seed treatments. Wheat treated with Dividend XL® RTA® and inoculated with *A. lipoferum* had the greatest recovery at each dilution time. Therefore, both diazotroph species appeared to be compatible with the fungicide treatment.

6.3 Nitrogen fixation and uptake

Some studies have found that the benefit from free-living N_2-fixers may be reduced where fertilizer-N is provided (Boddey et al., 1995; Kennedy and Islam, 2001). Furthermore, growth of the endophytic and epiphytic diazotrophs involved may also be repressed so that BNF is no longer possible with fertilizer-N applications. These growth chamber results showed N_2-fixation occurring with seed application of a liquid inoculant, containing either *A. lipoferum* or *G. azotocaptans*, at the 5-leaf and mature harvest times. There was also a significant ($P<0.05$) increase in N_2-fixation with the addition of fertilizer at those harvest times. Pots that received 12.2 and 24.5 µg N g^{-1} had similar %Ndfa and were significantly ($P<0.05$) higher than %Ndfa in pots with no fertilization.

Outcomes were comparable for the field experiment, where N_2-fixation was occurring at the flag-leaf and mature harvest times, for wheat inoculated with a liquid inoculant containing either *A. lipoferum* or *G. azotocaptans.* Significant ($P<0.05$) differences due to fertilization were

seen at the mature harvest time, and for analysis of heads and stems separately. As fertilizer levels increased, so did the %Ndfa. Plots that received 80 kg N ha^{-1} had the highest %Ndfa which was significantly (P<0.05) higher than plots that received no fertilizer. Based on the growth chamber and field experiments, fertilization did not reduce %Ndfa, in fact it enhanced it.

Nitrogen uptake followed the same general pattern as %Ndfa. As fertilizer levels increased, so did the amount of N taken up into the wheat plant. This was seen in both the growth chamber experiment as well as in the field experiment. There were significant (P<0.05) differences in N uptake in the growth chamber experiment from the inoculant. The most noteworthy finding was at the mature harvest. Analysis of total mature biomass showed that *A. lipoferum* and *G. azotocaptans* had significantly (P<0.05) higher N uptake over the control treatment. Analysis of the heads and stems separately showed that wheat treated with *A. lipoferum* had significantly (P<0.05) higher N uptake in heads than the other two treatments, and wheat treated with *G. azotocaptans* had significantly (P<0.05) higher N uptake in the stems than the other two treatments.

6.4 Further Research

6.4.1 Co-inoculation

Co-inoculation with symbiotic microorganisms, to create a successful system of free-living N_2-fixation in non-legume crops, can lead to some profits for plants. A notable characteristic of *Azospirillum* inoculation is that inoculation is more successful and more profitable when other microorganisms are co-inoculated with *Azospirillum* (Bashan and Holguin, 1997a, b). Dual benefits through increased N nutrition, via N_2-fixation, coupled with the production of plant growth hormones, perhaps aids the growth of each organism by synergistically providing nutrients, removing inhibitory products, and enhancing the ability of plants to grow. There are several groups of organisms that are known to fix N, but also produce phyto-hormones and/or provide protection against fungal and bacterial pathogens (Dobbelaere et al., 2003; Vessey, 2003; Ahmad et al., 2006). Studies have shown absorption of more nutrients by wheat plants occured because co-inoculation with diazotroph species provided access to more soil volume as root surface area was increased (Manske, 1990; Manske et al., 1995; Bahrani et

al., 2010). Other results have indicated that co-inoculation of wheat seeds with *A. brasilense* and *Rhizobium meliloti* had positive and significant effects on the grain yield and N, phosphorus (P) and potassium (K) content of the wheat grains compared to either single inoculation or control plants (Askary et al., 2009).

Further research into the effects of co-inoculation of *A. lipoferum* and *G. azotocaptans* on wheat could bring some insight into increasing productivity. Results from this growth chamber study showed that *A. lipoferum* was able to increase N uptake to the heads of wheat plants, whereas *G. azotocaptans* was able to increase N uptake to the stems of wheat plants. If further research produced similar results, combining the diazotroph species in an inoculant could provide many benefits. It has become common knowledge that early-season N application influences both yield and grain protein concentration while N application near or after anthesis influences only grain protein concentration (Wuest and Cassman, 1992; Ayoube et al., 1994; Rawluk et al., 2000; Woolfolk et al., 2002). Based on this past research, and the findings that *A. lipoferum* provides benefits to the heads and *G. azotocaptans* provides benefits to the stems of wheat plants, a co-inoculation could increase yield and protein content of grain, as well as increasing biomass. Increased biomass also influences and increases photosynthesis, higher grain yields, and more N in the straw could be returned to the soil at harvest.

6.4.2 Plant genotypes

There is a wide variability in yield responses of crops to diazotroph inoculation, thus supporting the concept that the genetic make-up of the host plant plays an important role in the establishment of a successful associative symbiosis between plants and organisms (Baldani and Dobereiner, 1979; Avivi and Feldman, 1982; Inigues et al., 2004). Differences in associative N_2-fixation have been observed between different lines of rice (Knauth et al., 2005), wheat (Neal and Larson, 1976; Santa, et al., 2004), maize and sorghum (Krotzky et al., 1986; Werner et al., 1989) and many other species.

Historically, plant breeders have not selected directly for interaction with beneficial soil bacteria, yet in Brazil, sugar cane breeders selecting for high yield under low-input conditions inadvertently selected for interaction with native diazotrophic bacteria (Baldani et al., 2002). Prior to the use of chemical fertilizers that have high plant available N, breeding programs of

other graminaceous crops like wheat, may also have indirectly selected for this association (Wissuwa et al., 2009). However, after decades of cultivar selection, under conditions that utilize chemical fertilizers, modern cultivars may not interact efficiently with these bacteria. Efforts are now focused on reintroducing these traits into cultivated wheat varieties (Fillery, 2007; Subbarao et al., 2007). Further support for this notion has been found in rice. Knauth et al. (2005) examined the composition of diazotrophic communities associated with related rice cultivars (*Oryza sativa*) and wild species (*Oryza brachyantha*) and found that when grown under identical conditions in the same soil without N fertilizer, there were remarkable differences in root associated *nif*H-gene expressing communities. Furthermore, *nif*H fragments expressed in the wild species of rice roots indicated that the active diazotrophs were not related to cultured strains.

For the intensive use of inoculants with associative bacteria, a wide isolation is needed, to select inside of the great existent diversity, the best combination between genotype of the plant and bacteria strain, as well as, the selection of efficient bacteria in the colonization of the roots, nitrate reducer, producer of phytohormones, and capable to fix significant amounts of N (Baldani et al., 2002; Santa et al., 2004). *Azospirillum lipoferum* is extensively tested, however very little is known about *G. azotocaptans* and its relationship with wheat as a host plant.

Future research regarding the interaction of *G. azotocaptans* and the more common wheat cultivars grown in western Canada (e.g., Superb) would give a better understanding of the most appropriate inoculant strain/plant genotype. In this current study Lillian wheat was used because of its solid stem characteristics and its ability to supress wheat stem sawfly damage. However this cultivar may not have been the best choice for *A. lipoferum* and *G. azotocaptans* used as inoculants. Perhaps the solid stem characteristics inhibited endophytic characteristics of the diazotrophs. For that reason, a study assessing how endophytic these diazotroph species were within the Lillian wheat would have been beneficial. In fact, assessment as to how endophytic *A. lipoferum* or *G. azotocaptans* are with any wheat plant would be beneficial. Also a study looking at the ability of early cereal varieties to utilize BNF compared with our current higher yielding varieties would be important, similar to the Brazilian sugar cane research. If past varieties were more favorable to endophytic colonization, as well as BNF activities, perhaps those characteristics could be added to current wheat varietal research.

There are many steps yet to take before *A. lipoferum* or *G. azotocaptans* would be ready as commercial inoculants. However, results found in this study show some promise to their use to reduce N fertilizer for wheat growth.

7. LITERATURE CITED

Abbadie, K., A. Mariotte, and J. Menaut. 1992. Independence of savanna grasses from soil organic matter for their nitrogen supply. Ecology 73:608-613.

Adesemoye, A.O., and J.W. Kloepper. 2009. Plant-microbes interactions in enhanced fertilizer-use efficiency. Appl. Microbiol. Biotechnol. 85:1-12.

Ahmad, F., I. Ahmad, F. Aqil, A. A. Wani, and Y. Sousche. 2006. Plant growth promoting potential of free-living diazotrophs and other rhizobacteria isolated from Northern Indian soil. Biotechnol. J. 1:1112-1123.

Ahmad, M., and M.S. Khan. 2010. Influence of selective herbicides on plant growth promoting traits of phosphate solubilizing *Enterobacter asburiae* strain PS2. Res. J. Microb. 5:849-857.

Alam, M., Z. Cui, T. Yamagishi, and R. Ishi. 2001. Grain yield and related physiological characteristics of rice plants (*Oryza sativa* L.) inoculated with free-living rhizobacteria. Plant Product. Sci. 4:126-130.

Alexander, D.B., and D.A. Zuberer. 1989. $^{15}N_2$ fixation by bacteria associated with maize roots at a low partial O_2 pressure. Appl. Environ. Microbiol. 55:1748-1753.

Allison, F., and C. Klein. 1962. Rates of immobilization and release of nitrogen following additions of carbonaceous materials and nitrogen to soils. Soil Sci. 93:383-386.

Aly, S., S. Soliman, E. El-Akel, and M. Ali. 1999. Significance of free N_2-fixing bacteria and nitrification inhibitors on saving the applied nitrogen to wheat plants. Bulletin of Faculty of Agriculture, University of Cairo. 50:347-365.

Andrews, M., E. James, S. Cummings, A. Zavalin, L. Vinogradova, and B. McKenzie. 2003. Use of nitrogen fixing bacteria inoculants as a substitute for nitrogen fertilizer for dryland graminaceous crops: Progress made, mechanisms of action and future potential. Symbiosis 35:209-229.

Angert, A., B. Luz, and D. Yakir. 2001. Fractionation of oxygen isotopes by respiration and diffusion in soils and its implications for the isotopic composition of atmospheric O_2. Global Biogeochem. Cycles 15:871-880.

Angus, J.F. 2001. Nitrogen supply and demand in Australian agriculture. Aust. J. Exp. Agric. 44:277-288.

Anitha, K.G., and M. Thangaraju. 2010. Influence of N fertilization on colonization and activity of *Gluconacetobacter diazotrophicus* in sugarcane. J. Agro. Crop Sci. 1:6-10.

Anyia, A.O., D.J. Archambault, C.J. Becquer, and J.J. Slaski. 2009. Plant growth-promoting diazotrophs and productivity of wheat on the Canadian prairies. In: M.S. Khan et al., editors, Microbial strategies for crop improvement. Springer Berlin Heidelberg. p.287-300.

Anyia, A.O., D.J. Archambault, and J.J. Slaski. 2004. Growth promoting effects of the diazotroph *Azorhizobium caulinodans* on Canadian wheat cultivars. Proceedings of the 4th International Crop Science Congress, Brisbane, Australia, p.201-202. www.cropscience.org.au/icsc2004/poster/2/5/6/863_anyiaa.htm

Arencibia, A.D., F. Vinagre, Y. Estevez, A. Bernal, J. Perez, J. Cacalcanti, I. Santana, and A.S. Hemerly. 2006. *Gluconacetobacter diazotrophicus* elicits a sugarcane defense response against a pathogenic bacteria *Xanthomonas albilineans.* Plant Signal. Behav. 1:265-273.

Armstrong, E.L., J.S. Pate, and D. Tennent. 1994. The field pea crop in south Western Australia – patterns of water use and root growth in genotypes of contrasting morphology and growth habit. Aust. J. Plant Physiol. 21:517-532.

Askary, M., A. Mostajeran, R. Amooaghaei and M. Mostajeran. 2009. Influence of the co-inoculation *Azospirillum brasilense* and *Rhizobium meliloti* plus 2,4-D on grain yield and N, P, K content of *Triticum aestivum* (cv. Baccros and Mahdavi). Am-Euras. J. Agric. Environ. Sci. 5:296-307.

Avivi, Y., and M. Feldman. 1982. The response of wheat to bacteria of the genus *Azospirillum*. Isr. J. Bot. 31:237-245.

Ayoub, M., S. Guertin, J. Fregeau-Reid, and D.L. Smith. 1994. Nitrogen fertilizer effect on breadmaking quality of hard red spring wheat in eastern Canada. Crop Sci. 34:1346-1352.

Azam, F., R. Mulvaney, and F. Stevenson. 1988. Quantification and potential availability of non-symbiotically fixed ^{15}N in soil. Biol. Fert. Soils 7:32-38.

Bahrani, A., J. Pourreza, and M.H. Joo. 2010. Response of winter wheat to co-inoculation with *Azotobacter* and arbescular mycorrhizal fungi (AMF) under different sources of nitrogen fertilizer. Am-Euras. J. Agric. Environ. Sci. 8:95-103.

Bais, H.P., T.L. Weir, L.G. Perry, S. Gilroy, and J.M. Vivanco. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Ann. Rev. Plant Biol. 57:233-266.

Balachandar, D., K. Kumar, and S. Kannaiyan. 2004. Evaluating nitrogen transfer efficiency of immobilized cyanobacteria to rice seedlings by ^{15}N technique. Int. Rice Res. Notes 29:53-54.

Baldani, J.I., and V.L.D. Baldani. 2005. History on the biological nitrogen fixation research in *graminaceous* plants: special emphasis on the Brazilian experience. An. Acad. Bras. Cienc. 77:549-579.

Baldani, J.I., L. Caruso, V.L.D. Baldani, S. Goi, and J. Döbereiner. 1997. Recent advances in BNF with non-legume plants. Soil Biol. Biochem. 29:911-922.

Baldani, J.I., V. Reis, V.L.D. Baldani, and J. Döbereiner. 2002. A brief story of nitrogen fixation in sugarcane-reasons for success in Brazil. Funct. Plant Biol. 29:417-423.

Baldani, V.L.D., J.I. Baldani, and J. Döbereiner. 1987. Inoculation of field-grown wheat (*Triticum aestivum*) with *Asospirillum* spp. in Brazil. Biol. Fert. Soils. 4:37-40.

Baldani, V.L.D., and J. Döbereiner. 1979. Host-plant specificity in the infection of cereals with *Azospirillum* spp. Soil Biol. Biochem. 12:433-439.

Baldani, V.L.D., E. James, J.I. Baldani, and J. Döbereiner. 1993. Colonization of rice by the nitrogen fixing bacteria *Herbaspirillum* spp. and *Azospirillum brasilense*. In:R. Palacios et al., editors, New horizons in nitrogen fixation. Kluwer Academic Publishers, The Netherlands. p.705-707.

Ball, B.C., I. Bingham, R.M. Rees, C.A. Watson, and A. Litterick. 2005. The role of crop rotations in determining soil structure and crop growth conditions. Can. J. Soil Sci. 85:557-577.

Barder, M., and D. Crawford. 1981. Effects of carbon and nitrogen supplementation on lignin and cellulose decomposition by a *Streptomyces*. Can. J. Microbiol. 27:859-863.

Barea, J.M., R. Azcón, and C. Azcón-Aguilar. 1983. Interactions between phosphate solubilizing bacteria and VA mycorrhiza to improve plant utilization of rock phosphate in non-acid soils. 3rd International Congress on Phosphorus Compounds, Brussels. p.127-144.

Barer, M., and R. Harwood. 1999. Bacterial viability and culturability. Adv. Microb. Physiol. 41:93-137.

Bashan, Y. 1986. Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. Soil Biol. Biochem. 18:297-301.

Bashan, Y. 1993. Potential use of *Azospirillum* as Biofertilizer. Turrialba. 43:286-291.

Bashan, Y. 1998. Inoculants of plant growth-promoting bacteria for use in agriculture. Biotechnol. Adv. 16:729-770.

Bashan, Y., and G. Holguin. 1997a. *Azospirillum*-plant relationships: environmental and physiological advances (1990-1996). Can. J. Microbiol. 43:103-121.

Bashan, Y., and G. Holguin. 1997b. Short and medium-term avenues for *Azospirillum* inoculation. In: A. Ogoshi et al., editors, Plant growth promoting rhizobacteria – present status and future prospects. Faculty of Agriculture, Hokkaido University, Sapporo. p.130-149.

Bashan, Y., G. Holguin, and L. de-Bashan. 2004. *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). Can. J. Microbiol. 50:521-577.

Bashan, Y., and H. Levanony. 1988. Adsorption of the rhizosphere bacterium *Azospirillum brasilense* Cd to soil, sand and peat particles. J. Gen. Microbial. 134:181 l-1820.

Bashan, Y., and H. Levanony. 1989. Factors affecting adsorption of *Azospirillum brasilense* Cd to root hairs as compared with root surface of wheat. Can. J. Microbiol. 35:936-944.

Bashan, Y., and H. Levanony. 1990. Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture. Can. J. Microbiol. 36:591-608.

Bashan, Y., M.E. Puente, M.N. Rodriguez-Mendoza, G. Toledo, G. Holguin, R. Ferrera-Cerrato, and S. Pedrin. 1995. Survival of *Azospirillum brasilense* in the bulk soil and rhizosphere of 23 soil types. Appl. Environ. Microbiol. 61:1938-1945.

Belimov, A., A. Kunakova, N. Vasilyeva, E. Gruzdeva, N. Vorobiev, A. Kojemiakov, O. Khamova, S. Postavskaya, and S. Sokova. 1995. Relationship between survival rates of associative nitrogen-fixers on roots and yield response of plants to inoculation. FEMS Microbiol. Ecol. 17:187-196.

Belnap, J. 2001. 19 factors influencing nitrogen fixation and nitrogen release in biological soil crusts. Eco. Studies. 150:241-261.

Bergersen, F.J. 1980. Measurement of nitrogen fixation by direct means. In: F. Bergersen, editor, Methods for evaluating bioligical nitrogen fixation. Wiley, New York. p.65-110.

Bergersen, F.J. 1991. Physiological control of nitrogenase and uptake hydrogenase. In: M. Dilworth, and A Glenn, editors, Biology and biochemistry of nitrogen fixation. Elsevier, Amsterdam. p.76-102.

Bertin, C., X. Yang, and L.A. Weston. 2003. The role of root exudates and allelochemicals in the rhizosphere. Plant Soil. 256:67-83.

Bhattacharjee, R.B., A. Singh, and S.N. Mukhopadhgay. 2008. Use of nitrogen-fixing bacteria as biofertilizer for non-legumes: prospects and challenges. Appl. Micro. Biotech. 80:199-209.

Bhuiyan, N.I. 1995. Intensive cropping and soil nutrient balance in Bangladesh. In: M. Hussain et al., editors, Improving soil management for intensive cropping in the tropics and sub-tropics. Bangladesh Agricultural Research Council, Dhaka. p.61-67.

Biggs, I., J. Wilson, B. Keating, and C. Critchley. 2000. Does biological N_2-fixation contribute to nitrogen requirements in Australian sugarcane? Proceedings of the Austalian Society for Sugar Cane Technology. 22:133-138.

Bloemberg, G. 2007. Microscopic analysis of plant-bacterium interactions using auto fluorescent proteins. Euro. J. Plant Pathol. 199:301-309.

Boddey, R.M. 1987. Methods for quantification of nitrogen fixation associated with granineae. CRC Critical Rev. Plant Sci. 6:209-266.

Boddey, R.M., B.J.R. Alves, and S. Urguiaga. 1998. Evaluation of biological nitrogen fixation associated with non-legumes. Dev. Plant Soil Sci. 79:287-305.

Boddey, R.M., P.M. Chalk, R.L. Victoria, and E. Matsui. 1983. The ^{15}N-isotope dilution technique applied to the estimation of biological nitrogen fixation associated with *Paspalum notatum* cv. Batatais in the field. Soil Biol. Biochem. 15:25-32.

Boddey, R.M., O. de Oliveira, S. Urquiaga, M. Reis, F. de Olivares, V. Baldani, and J. Döbereiner. 1995. Biological nitrogen fixation associated with sugar cane and rice: Contributions and prospects for improvement. Plant Soil. 174:195-209.

Boddey, R.M., M. Peoples, B. Palmer, and P. Dart. 2000. Use of the ^{15}N natural abundance technique to quantify biological nitrogen fixation by woody perennials. Nutr. Cycl. Agroecosys. 57:235-270.

Boddey, R.M., J. Polidoro, A. Resende, B. Alves, and S. Urquiaga. 2001. Use of the ^{15}N natural abundance technique for the quantification of the contribution of N_2 fixation to sugar cane and other grasses. Aust. J. Plant Phys. 28:889-895.

Boddey, R.M., and R.L. Victoria. 1986. Estimation of biological N_2-fixation associated with *Brochiaria* and *Paspalm notatum* using ^{15}N labelled organic matter and fertilizer. Plant Soil. 90:265-292.

Bouton, J.H., S. Albrecht, and D. Zuberer. 1985. Screening and selection of pearl millet for root associated bacterial nitrogen fixation. Field Crops Res. 11:131-140.

Brouzes, R., J. Lasik, and R. Knowles. 1969. The effect of organic amendment, water content, and oxygen on the incorporation of $^{15}N_2$ by some agriculture and forest soils. Can. J. Microbiol. 15:899-905.

Buckley, D.H., V. Huangyutitham, S.F. Hsu, and T.A. Nelson. 2007. Stable isotope probing with $^{15}N_2$ reveals novel noncultivated diazotrophs in soil. App. Environ. Microbiol. 73:3196-3204.

Buckley, D.H., V. Huangyutitham, S.F. Hsu, and T.A. Nelson. 2008. $^{15}N_2$-DNA-stable isotope probing of diazotrophic methanotrophs in soil. Soil Biol. Biochem. 40:1272-1283.

Buckley, D.H., and T.M. Schmidt. 2003. Diversity and dynamics of microbial communities in soils from agroecosystems. Environ. Microbiol. 5:441-452.

Burdman, S., E. Jurkevitch, and Y. Okon. 2000. Recent advances in the use of plant growth promoting rhizobacteria (PGPR) in agriculture. In: N.S. Rao, and Y.R. Dommergues, editors, Microbial interactions in agriculture and forestry (Volume II). Science Publishers Inc. Plymouth, UK. p.229-250.

Burris, R., F. Eppling, H. Wahlin, and P. Wilson. 1943. Detection of nitrogen fixation with isotopic nitrogen. J. Biol. Chem. 148:349-357.

Caballero-Mellado, J., M. Carcano-Montiel, and M. Mascarua-Esparza. 1992. Field inoculation of wheat (*triticum aestivum*) with *Azospirillum brasilense* under temperate climate. Symbiosis. 13:243-253.

Campbell, C.A., H.R. Davidson, and F.G. Warder. 1977. Effects of fertilizer N and soil moisture on yield components, protein content and N accumulation in the aboveground parts of spring wheat. Can. J. Soil Sci. 57:311-327.

Cato, E., W. George, and S. Finegold. 1986. Genus *Clostridium*. In: P. Sneath et al., editors, Bergey's manual of systematic bacteriology. Williams and Wilkins, Baltimore, MD. p.1141-1200.

Cavalcante, V.A., and J. Dobereiner. 1988. A new acid-tolerant nitrogen fixing bacterium associated with sugarcane. Plant Soil. 8:23-31.

Chalam, A.V., C. Sasikala, C.V. Ramana, N.R. Uma, and P.R. Rao. 1997. Effect of pesticides on the diazotrophic growth and nitrogenase activity of purple nonsulfur bacteria. Bull Environ. Contam. Toxicol. 58:463-468.

Charles, R., G. Jolliet, and D. Pellet. 2006. Environmental analysis of intensity level in wheat crop production using life cycle assessment. Agric. Ecosyst. Environ. 113:216-225.

Chaulk, P.M. 1985. Estimation of N_2 fixation by isotope dilution: An appraisal of techniques involving ^{15}N enrichment and their application. Soil Biol. Biochem. 17:389-410.

Chaulk, P.M. 1991. The contribution of associative and symbiotic nitrogen fixation to the nitrogen nutrition of non-legumes. Plant Soil. 132:29-39.

Chelius, M.K., and E.W. Triplett. 2000. Diazotrophic endophytes associated with maize. In: E.W. Triplett, editor, Prokaryotic nitrogen fixation: a model system for the analysis of a biological process. Horizon Scientific Press, Norfolk, UK. p.779-792.

Christiansen-Weniger. C. 1998. Endophytic establishment of diazotrophic bacteria in auxin-induced tumors of cereal crops. Crit. Rev. Plant Sci. 17:55-76.

Cleveland, C., A. Townsend, D. Schimel, H. Fisher, R. Howard, L. Hedin, S. Perakis, E. Latty, J. Von Fischer, A. Elseroad, and M. Wasson. 1999. Global patterns of terrestrial biological nitrogen (N2) fixation in natural ecosystems. Global Biogeochem. Cycl. 13:623-645.

Clogg, C.C., E. Petkova, and A. Haritou. 1995. Statistical methods for comparing regression coefficients between models. Am. J. Soc. 100:1261-1293.

Cocking, E.C. 2002. Concerted action for cereal and other non-legume crop nitrogen fixation, enhanced growth and yield. In: I.R. Kennedy, and A.T.M.A. Choudhury, editors, Biofertilisers in action. Rural Industries Research and Development Corporation, Canberra, AU. p.1–3.

Cocking, E.C. 2003. Endophytic colonization of plant roots by nitrogen-fixing bacteria. Plant and Soil. 252:169-175.

Cohen, E., Y. Okon, J. Kigel, I. Nur, and Y. Henis. 1980. Increase in dry weight and total nitrogen content in *Zea mays* and *Setaria italic* associated with nitrogen-fixing *Azospirillum* spp. Plant Physiol. 66:746-749.

Colnaghi, R., A. Green, L. He, P. Rudnick, and C. Kennedy. 1997. Strategies for increased mmonium production in free-living or plant associated nitrogen fixing bacterial. Plant Soil. 194:145-154.

Conklin, A., and P. Biswas. 1978. A survey of asymbiotic nitrogen fixation in the rhizosphere of weeds. Weed Sci. 26:148-150.

Cookson, W.R., D.V. Murphy, and M.M. Roper. 2008. Characterizing the relationships between soil organic matter components and microbial function and composition along a tillage disturbance gradient. Soil Biol. Biochem. 40:763-777.

Creus, D.M., R.J. Sueldo, and C.A. Barassi. 1996. *Azospirillum* inoculation in pregerminating wheat seeds. Can. J. Microbiol. 42:83-86.

Curley, R.L., and J.C. Burton. 1975. Compatibility of *Rhizobium japonicum* with chemical seed protectants. Agron. J. 67:807-808.

Cycon, M., Z. Piotrowska-Seget, A. Kaczynska, and J. Kozdroj. 2006. Microbiological characteristics of a sandy loam soil exposed to tebuconazole and λ-cyhalothrin under laboratory conditions. Ecotoxicol. 15:639-646.

Dalton, H. 1980. The cultivation of diazotrophic microorganisms. In: F. Bergersen, editor, Methods for evaluating biological nitrogen fixation. Wiley, Chichester, UK. p.13-64.

Danso, S.K.A., G. Hardarson, and F. Zapata. 1993. Misconceptions and practical problems in the use of ^{15}N soil enrichment techniques for estimating N_2 fixation. Plant Soil. 152:25-52.

Dardanelli, M.S., F.J.F. de Córdoba, M.R. Espuny, M.A.R. Carvajal, M.E.S. Diaz, A.M.G. Serrano, Y. Okon, and M. Megias. 2008. Effect of *Azospirillum brasilense* coinoculated with *Rhizobium* on *Phaseolus vulgaris* flavonoids and Nod factor production under salt stress. Soil Biol. Biochem. 40:2713-2721.

Dart, P. 1986. Nitrogen fixation associated with non-legumes in agriculture. Plant Soil. 90:303-334.

Das, A., and D. Saha. 2003. Influences of diazotrophic inoculations on nitrogen nutrition of rice. Aust. J. Soil Res. 41:1543-1554.

de Campos, S., L. Roesch, M. Zanettini, and L. Passaglia. 2006. Relationship between in vitro enhanced nitrogenase activity of an *Azospirillum brasilense* Sp7 mutant and its growth-promoting activities *in situ*. Current Microbiol. 53:43-47.

de Carvalho, A., B. Alves, V. Baldani, and V. Reis. 2008. Application of ^{15}N natural abundance technique for evaluating biological nitrogen fixation in oil palm ecotypes at nursery stage in pot experiments and at mature plantation sites. Plant Soil. 302:71-78.

del Gallo, M., and P. Fabri. 1991. Effect of soil organic matter on chickpea inoculated with *Azospirillum brasilense* and *Rhizobium leguminosarum* bv. *ciceri.* Plant Soil. 137:171-175.

de Salamone, I.E.G., J. Döbereiner, S. Urquiaga, and R.M. Boddey. 1996. Biological nitrogen fixation in *Azospirillum* strain-maize genotype associations as evaluated by ^{15}N isotope dilution technique. Biol. Fertil. Soils. 23:249-256.

Denef, K., J. Six, H. Bossuyt, S. Frey, E. Elliott, R. Merckx, and K. Paustian. 2001. Influence of dry-wet cycles on the interrelationship between aggregate, particulate organic matter, and microbial community dynamics. Soil Biol. Biochem. 33:1599-1611.

Diaz-Zorita, M., and M.V. Fernandez-Canigia. 2008. Field performance of a liquid formulation of *Azospirillum brasilense* on dryland wheat productivity. Eur. J. Soil Biol. 1:1-9.

Di Ciocco, C.A., and E.R. Caceres. 1997. Effect of the fungicide captan on *Azospirillum brasilense* CD in pure culture and associated with *Setaria italica*. Rev. Argent. Microbiol. 29:152-156.

Dilworth, M.J. 1974. Dinitrogen fixation. Annu. Rev. Plant Physiol. 25:81-114.

Döbereiner, J. 1995. Isolation and identification of aerobic nitrogen-fixing bacteria from soil and plants. In: K. Alef K, and P Nannipieri, editors, Methods in applied soil microbiology and biochemistry. Academic, London, UK. p.134-141.

Dobbelaere, S., A. Croonenborghs, A. Thys, D. Ptacek, J. Vanderleyden, P. Dutto, C. Labandera-Gonzalez, J. Cballero-Mellado, J. Aguirre, Y. Kapulnik, S. Brener, S. Burdman, D. Kadouri, S. Sarig, and Y. Okon. 2001. Responses of agronomically important crops to inoculation with *Azospirillum*. Aust. J. Plant Physiol. 28:871-879.

Dobbelaere, S., and Y. Okon. 2003. The plant growth promoting effect and plant responses. In: C. Elmerich, and W.E. Newton, editors, Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations. Kluwer Academic. The Netherlands. p.1-26.

Dobbelaere, S., J. Vanderleyden, and Y. Okon. 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit. Rev. Plant Sci. 22:107-149.

Eady, R. 1980. Methods for studying nitrogenase. In: F. Bergersen, editor, Methods for evaluating biological nitrogen fixation. Wiley, Chichester, UK. p.213-264.

Eisele, K.A., D.S. Schimel, L.A. Kapustka, and W.J. Parton. 1989. Effects of available P and N:P ratios on non-symbiotic dinitrogen fixation in tallgrass prairie soils. Oecologia. 79:471-474.

Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, H. Mitsui, and K. Minamisawa. 2001. Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. 67:5285-5293.

Elhai, J., and C. Wolk. 1990. Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacterium *Anabaena*. EMBO J. 9:3379-3388.

El-Komy, H.M.A. 2005. Coimmobilization of *Azospirillum lipoferum* and *Bacillus megaterium* for successful phosphorus and nitrogen nutrition of wheat plants. Food Technol. Biotechnol. 43:19-27.

El Zemrany, H., J. Cortet, M.P. Lutz, A. Chabert, E. Baudoin, J. Haurat, N. Maughan, D. Felix, G. Defago, R. Bally, and Y. Moenne-Loccoz. 2006. Field survival of the phytostimulator *Azospirillum lipoferum* CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilization. Soil Biol. Biochem. 38:1712-1726.

Evans, J., A.M. McNeill, M.J. Unkovich, N.A. Fettell, and D.P. Heenan. 2001. Net nitrogen balances for cool-season grain legume crops and contributions to wheat nitrogen uptake: a review. Aust. J. Exp. Agric. 41:347-359.

Fages, J. 1992. An industrial view of *Azospirillum* inoculants: formulation and application technology. Symbiosis 13:15-26.

Ferreira, J.S., J.I. Baldani, and V.L.D. Baldani. 2010. Selection of peat-based inoculants containing diazotrophs in two varieties of rice. Acta. Sci. Agron. 32:179–185.

Ferreira, J.S., D.C.C. Sabino, S.L. Guimarães, J.I. Baldani, and V.D.L. Baldani. 2003. Selection vehicles for the preparation of inoculant bacteria with diazotrophic for flooded rice. Rev. Agron. 37:6–12.

Fillery, I. 2007. Plant-based manipulation of nitrification in soil: a new approach to managing N loss? Plant Soil. 294:1-4.

Francis, G.S., and T.L. Knight. 1993. Long-term effects of conventional and no-tillage on selected soil properties and crop yields in Canterbury, New Zealand. Soil Tillage Res. 26:193-210.

Frankenberger W, and M. Arshad. 1995. Phytohormones in Soils. Plant Growth Reg. 24:145

Fraser, P.M. 1994. The impact of soil and crop management practices on soil macrofauna. In: C.E. Pankurst et al., editors, Soil biota: Management in sustainable farming systems. CSIRO, Melbourne, AU. p.125-132.

Fried, M., and V. Middelboe. 1977. Measurement of amount of nitrogen fixed by legume crop. Plant Soil. 47:713-715.

Fried, M., S.K.A. Danso, and F. Zapata. 1983. The methodology of measurement of N_2 fixation by non-legumes as inferred from field experiments with legumes. Can. J. Microbiol. 29:1053-1062.

Gallo, M., and I. Fendrik. 1994. The rhizosphere and *Azospirillum*. In: Y. Okon, editor, *Azospirillum* plant associations. CRC Press, Boca Raton, Florida. p.57-75.

Gallori, E., E. Casalone, C.M. Colella, S. Daly and M. Polsinelli. 1991. 1, 8-naphthalic anhydride antidote enhances the toxic effects of captan and thiram fungicides on *Azospirillum bransilense* cells. Res. Microbiol. 142:1005-1012.

Gallori, E., E. Casalone, O. Nocita, L. Turbanti, and M. Polsinelli. 1988. Isolation and characterization of a glutathione-overproducing, captan-resistant mutant of *Azospirillum brasilense*. J. Gen. Microbiol. 134:3173-3178.

Garabet, S., J. Ryan and M. Wood. 1998. Nitrogen and water effects on wheat yield in a Mediterranean-type climate: II. Fertilizer-use efficiency with labeled nitrogen. Field Crop. Res. 58:213-221.

Gentry, T., G. Wickham, C. Schadt, Z. He, and J. Zhou. 2006. Microarray applications in microbial ecology research. Microb. Ecol. 52:159-175.

Giller, K. 1987. Use and abuse of the acetylene reduction assay for measurement of "associative" nitrogen fixation. Soil Biol. Biochem. 19:783-784.

Giller, K., J. Day, P. Dart, and S. Wani. 1984. A method for measuring the transfer of fixed nitrogen from free-living bacteria to higher plants using $^{15}N_2$. J. Microbiol. Methods. 2:307-316.

Giller, K., and R. Merckx. 2003. Exploring the boundaries of N_2-fixation in cereals and grasses: an hypothetical and experimental framework. Symbiosis. 35:3-17.

Giller, K., S. Wani, J. Day, and P. Dart. 1988. Short-term measurements of uptake of nitrogen fixed in the rhizospheres of sorgum (*Sorghum bicolor*) and millet (*Pennisetum americanum*). Biol. Fertil. Soils. 7:11-15.

Giller, K., and K. Wilson. 1993. Nitrogen Fixation in Tropical Cropping Systems. CAB International, Wallingford, UK.

Gomes, F., V. Salmeron, B. Rodelas, M.V. Martinez-Toledo, and J. Gonzalez-Lopez. 1998. Response of *Azospirillum brasilense* to the pesticides bromopropylate and methidathion on chemically defined media and dialysed-soil media. Ecotoxicol. 7:43-47.

Gray, E.J., and D.L. Smith. 2005. Intracellular and extracellular PGPR: Commonalities and distinctions in the plant-bacterium signaling processes. Soil Biol. Biochem. 37:395-412.

Greenland, D. 1971. Changes in the nitrogen status and physical condition of soils under pastures, with special reference to the maintenance of the fertility of Australian soils used for growing wheat. Soils Fert. 34:237-251.

Griggs, B.R., R.J. Norman, and W. Johnson. 2002. Influence of nitrogen fertilization rate on the uptake of ^{15}N labeled urea and ammonium sulfate by soft red winter wheat in the presence and absence of *Poa annua*. p.106-115. http://www.*arkansaswheat.org/research_results/pdf/influenceofnitrogen.pdf*

Gupta, V., M. Roper, and D. Roget. 2006. Potential for non-symbiotic N_2-fixation in different agroecological zones of southern Australia. Aust. J. Soil Res. 44:343-354.

Hadar, Y. 1986. The role of organic matter in the introduction of biofertilizers and biocontrol agents to soils. In: Y. Chen, editor, The role of organic matter in modern agriculture. Martinus Nijhof, Dordrecht, UK. p.169-179.

Halsall, D.M., G.L. Turner, and A.H. Gibson. 1985. Straw and xylan utilization by pure cultures of nitrogen-fixing *Azospirillum* spp. Appl. Environ. Microbiol. 49:423-428.

Halvorson, A.D., R.F. Follett, M.E. Bartolo, and F.C. Schweissing. 2002. Nitrogen fertilizer use efficiency of furrow-irrigated onion and corn. Agron. J. 94:442-449.

Hamelin, J., N. Fromin, S. Tarnawski, S. Teyssier-Cuvelle, and M. Aragno. 2002. *nif*H gene diversity in the bacterial community associated with the rhizosphere of *Molinia coerulea*, an oligonitrophilic perennial grass. Environ. Microbiol. 4:477-481.

Han, S.O., and P.B. New. 1998. Variation in nitrogen fixing ability among natural isolates of *Azospirillum.* Microbial Ecol. 36:193-201.

Hardarson, G., and S.K.A. Danso. 1990. Use of ^{15}N methodology to assess biological nitrogen fixation. In: G. Hardson, editor, Use of nuclear techniques in studies of soil-plant relationships. Training course series no. 2. International Atomic Energy Agency, Vienna, Austria. p.129-160.

Hardy, R., R.L. Burns and R.O. Holsten. 1973. Applications of the acetylene-reduction assay for measurement of nitrogen fixation. Soil Biol. Biochem. 5:47-81.

Hardy, R., R.O. Holsten, E. Jackson, and R. Burns. 1968. The acetylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. Plant Phys. 43:1185-1207.

Harris, J.M., J.A. Lucas, M.R. Davey, G. Lethbridge, and K.A. Powell. 1989. Establishment of *Azospirillum* inoculant in the rhizosphere of winter wheat. Soil Biol. Biochem. 21:59-64.

Hashem, F.M., S.A. Saleh, V. Berkum, and M. Voll. 1997. Survival of *Bradyrhizobium sp. (Arachis)* on fungicide-treated peanut seed in relationship to plant growth and yield. World J. Microbiol. Biotechnol. 13:335-340.

Havelka, U., M. Boyle, and R. Hardy, 1982. Biological nitrogen fixation. In: J. Stevenson, editor, Nitrogen in agricultural soils. Agronomy Monograph No. 22, ASA-CSSA-SSSA, Madison, Wisconsin, p.365-422.

Hayat, R., S. Ali, U. Amara, R. Khalid, and I. Ahmed. 2010. Soil beneficial bacteria and their role in plant growth promotion: A review. Ann. Microbiol. 60:579-598.

He, Z., T. Gentry, C. Schadt, L. Wu, J. Liebich, S. Chong, Z. Huang, W. Wu, B. Gu, P. Jardine, C. Criddle, and J. Zhou. 2007. GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. The ISME J. 1:67-77.

Hirota, Y., T. Fujii, Y. Sano, and S. Iyama. 1978. Nitrogen fixation in the rhizosphere of rice. Nature. 276:416-417.

Hnatowich, G. 2000. Pulse production manual 2000. Saskatchewan Pulse Growers, Saskatoon, SK, Canada.

Hoagland, L., K. Murphy, L. Carpenter-Boggs, and S. Jones. 2008. Improving nutrient uptake in wheat through cultivar specific interaction with *Azospirillum*. Second Scientific Conference of the International Society for Organic Agricultural Research. p.526-565.

Hsu, S.F., and D.H. Buckley. 2009. Evidence for the functional significance of diazotroph community structure in soil. ISME J. 3:124–136.

Hurek, T., L. Handley, B. Reinhld-Hurek, and Y. Piché. 2002. *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Mol. Plant Microbe Interact. 15:233-242.

Huddedar, S.B., S.D. Gore, and B.A. Chopade. 2000. Studies on distribution and characterization of Acinetobacter genospecies isolated from rhizosphere of wheat exhibiting antifungal and antibacterial activity. In: Proceedings of International Conference on Microbial Biotechnology, Trade and Public Policy, Hyderabad, India. p.1-16.

IAEA. 2001. Manual on "use of isotope and radiation methods in soil and water management and crop nutrition." IAEA-TCS-14

Iniguez, A.L., Y. Dong, and E.W. Triplett. 2004. Nitrogen fixation in wheat provided by *Klebsiella pneumonia* 342. Mol. Plant Microbe Interact. 17:078-1085.

Jacoud, C., D. Job, P. Wadoux, and R. Bally. 1999. Initiation of root growth stimulation by *Azospirillum lipoferum* CRT1 during maize seed germination. Can. J. Microbiol. 45:339-342.

Jain, D.K., and D.G. Patriquin. 1984. Root hair deformation, bacterial attachment, and plant growth in wheat-*Azospirillum* associations. Appl. Environ. Microbiol. 48:1208-1213.

James, E. 2000. Nitrogen fixation in endophytic and associative symbiosis. Field Crop. Res. 65:197-209.

James, E., and F. Olivares. 1998. Infection and colonization of sugarcane and other graminaceous plants by endophytic diazotrophs. Crit. Rev. Plant Sci. 17:77-119.

James, E., F. Olivares, A.L.M. de Oliveira, F.D. dos Reis Jr., L.G. da Silva, and V.M. Reis. 2000. Further observations on the interaction between sugar cane and *Gluconacetobacter diazotrophicus* under laboratory and greenhouse conditions. J. Exp. Bot. 52:747-760.

Janssen, P. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Applied Environ. Microbiol. 72:1719-1728.

Janzen, H.H., and R.M.N. Kucey. 1988. C, N and S mineralization of crop residues as influenced by crop species and nutrient regime. Plant Soil. 106:35-41.

Jenkinson, D. 2001. The impact of humans on the nitrogen cycle, with focus on temperate arable agriculture. Plant Soil. 228:3-15.

Jensen, E., and H. Hauggaard-Nielsen. 2003. How can increased use of biological N_2 fixation in agriculture benefit the environment? Plant Soil. 252:177-186.

Jensen, V. 1981. Heterotrophic micro-organisms. In: W. Broughton, editor, Nitrogen fixation, Volume 1. Ecology. Clarendon Press, Oxford, UK. p.30-56.

Jones, D.L., C. Nguyen, and R.D. Finlay. 2009. Carbon flow in the rhizosphere: carbon trading at the soil-root interface. Plant Soil. 321:5-33.

Jones, K., and R. Wilson. 1978. The fate of nitrogen fixed by a free-living blue-green alga. Ecol. Bull. (Stockholm) 26:158-163.

Kapulnik, Y., Y. Okon, and Y. Henis. 1987. Yield response of spring wheat cultivars (*Triticum aestivum* and *T. turgidum*) to inoculation with *Azospirillum brasilense* under field conditions. Biol. Fertil. Soils. 4:27-35.

Kavadia, A., D.V. Vayenas, S. Pavlou , and G. Aggelis. 2007. Dynamics of free-living nitrogen-fixing bacterial populations in antagonistic conditions. Ecol. Modelling. 200:243-253.

Kennedy, I., A.T.M.A. Choudhury, and M. Kecskés. 2004. Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biol. Biochem. 36:1229-1244.

Kennedy, I., and N. Islam. 2001. The current and potential contribution of asymbiotic nitrogen fixation to nitrogen requirements on farms: a review. Aust. J. Exp. Agric. 41:447-457.

Kennedy, I., L.L. Pereg-Gerk, C. Wood, R. Deaker, K. Gilchrist, and S. Katupitiya. 1997. Biological nitrogen fixation in non-leguminous field crops: facilitating the evolution of an effective association between *Azospirillum* and wheat. Plant Soil. 194:65-79.

Kennedy, I., and Y. Tchan. 1992. Biological nitrogen fixation in non-leguminous field crops: Recent advances. Plant Soil. 141:93-118.

Kenney, D. 1997. Commercialization of biological control products in the chemical pesticide world. In: A. Ogoshi et al., editors, Plant growth-promoting rhizobacteria – present status and future prospects. Faculty of Agriculture, Hokkaido University, Sapporo, Japan. p.126-127.

Khan, A.A., G. Jibni, M.S. Akhtar, S.M.S. Naqvi, and M. Rasheed. 2009. Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. J. Agric. Biol. Sci. 1:48-58.

Kloepper, B., R.W. Rickman, and P. Chevalier. 1998. The physiological life cycle of wheat: Its use in breeding and crop management. Euphytica. 100:341-347.

Knauth, S., T. Hurek, D. Brar, and B. Reinhold-Hurek. 2005. Influence of different *Oryza* cultivars on expression of *nif*H gene pools in roots of rice. Environ. Microbiol. 7:1725-1733.

Knowles, R. 1980. Nitrogen fixation in natural plant communities and soils. In: F. Bergersen, editor, Methods for evaluating biological nitrogen fixation. John Wiley & Sons, Chichester, UK. p.557-582.

Kostychev, S., A. Sheloumova, and Shul'gina. 1926. Nitrogen content of soils of the southern coast of Crimea (*Russian*). Sonetskii Agron. 1. 1926.

Krotzky, A., R. Berggold, and D. Werner. 1986. Analysis of factors limiting associative N_2-fixation (C_2H_2-reduction) with two cultivars of *Sorghum nutans*. Soil Biol. Biochem. 18:201-207.

Krotzky, A., R. Berggold, and D. Werner. 1988. Plant characteristics limiting associative N_2-fixation (C_2H_2-reduction) with two cultivars of *Sorghum nutans*. Soil Biol. Biochem. 20:157-162.

Kuhner, C., C. Matthies, G. Acker, M. Schmittroth, A. Grossner, and H. Drake. 2000. *Clostridium akagii* sp. Nov. and *Clostridium acidisol* sp. Nov.: acid-tolerant, N_2-fixing clostridia isolated from acidic forest soil and litter. Int. J. Syst. Evol. Microbiol. 50:873-881.

Lamb, J.A., J.W. Doran, and G.A. Peterson. 1987. Nonsymbiotic dinitrogen fixation in no-till and conventional wheat-fallow systems. Soil Sci. Soc. Am. J. 51:365-361.

Large, E.G. 1954. Growth stages in cereals: illustration of the Feeke's scale. Plant Path. 3:128-129.

Lethbridge, G., and M.S. Davidson. 1983a. Root-associated nitrogen-fixing bacteria and their role in the nitrogen nutrition of wheat estimated by ^{15}N isotope dilution. Soil Biol. Biochem. 15:365-374.

Lethbridge, G., and M.S. Davidson. 1983b. Microbial biomass as a source of nitrogen for cereals. Soil Biol. Biochem. 15:375-376.

Lethbridge, G., M.S. Davidson, and G.P. Sparling. 1982. Critical evaluation of the acetylene reduction test for estimating the activity of nitrogen-fixing bacteria associated with the roots of wheat and barley. Soil Biol. Biochem. 14:27-35.

Li, C., J. Aber, F. Stange, K. Butterbach-Bahl, and H. Papen. 2000. A process-oriented model of N_2O and NO emissions from forest soils: I. Model development. J of Geophys. Res. 105:4369-4384.

Li, R., and I. Macrae. 1991. Specific association of diazotrophic acetobacters with sugarcane. Soil Biol. Biochem. 23:999-1002.

Lima, E., R. Boddey, and J. Döbereiner. 1987. Quantification of biological nitrogen fixation associated with sugar cane using a ^{15}N aided nitrogen balance. Soil Biol. Biochem. 19:165-170.

Line, M., and M. Loutit. 1973. Nitrogen-fixation by mixed cultures of aerobic and anaerobic micro-organisms in an aerobic environment. J. Gen. Microbiol. 74:179-180.

Lors, C., B. Lagacherie, C. Chabanet, and G. Souals. 2005. A mode pollutant, adversely affects the potential of soil microbial communities to mineralize the herbicide 2, 4-D: an investigation using micro-sampling procedures. Soil Biol. Biochem. 37:1023-1028.

Lugtenberg, B.J.J., T.F.C. Chin-A-Woeng, and G.V. Bloemberg. 2002. Microbe-plant interactions: principles and mechanisms. A. Van Leeuw. J. Microbiol. 81:373-383.

Madhuri, R.J., and V. Rangaswamy. 2003. Influence of selected fungicides on microbial population in groundnut (*Arachis hypogeae* L.) soils. Pollut. Res. 22:205-212.

Mahdi, S.S., G.I. Hassan, S.A. Samoon, H.A. Rather, S.A. Dar, and B. Zehra. 2010. Bio-fertilizers in organic agriculture. J. Phytol. 2:45-54.

Malik, K.A., B. Rakhshanda, S. Menhaz, G. Rasul, M.S. Mirza, and S. Ali. 1997. Association of nitrogen-fixing, plant-growth-promoting rhizobacteria (PGPR) with kallar grass and rice. Plant Soil. 194:37-44.

Malik, K.A., Y. Zafar, R. Bilal, and F. Azam. 1987. Use of ^{15}N isotope dilution for quantification of N_2-fixation associated with roots of kallar grass (*Leptochola fusca* (L.)). Biol. Fertil. Soils. 4:103-108.

Manske, G.G.B. 1990. Genetical analysis of the efficiency of VA mycorrhiza with spring wheat. 1.Genotypic differences and reciprocal cross between an efficient and non-efficient variety. In: N.E. Bassam et al., editors. Genetic aspects of plant mineral nutrition. Kluwer Academic Publishers. Dordrecht, Netherlands. p.397-405.

Manske, G.G.B., A.B. Luttger, R.K. Behl and P.L.G. Vlek. 1995. Nutrient efficiency based on VA mycorrhiza (VAM) and total root length of wheat cultivars grown in India. Angew. Bot. 69:108-110.

McCully, M. 2001. Niches for bacterial endophytes in crop plants: a plant biologist's view. Aust. J. Plant Physiol. 28:983-990.

Meeks, J., E. Campbell, and P. Bisen. 1994. Elements interrupting nitrogen fixation genes in cyanobacteria: presence and absence of a *nifD* element in clones of *Nostoc* sp. Strain Mac. Microbiol. 140:3225-3232.

Mehnaz, S., and G. Lazarovits. 2006. Inoculation effects of *Pseudomonas putida, Gluconacetobacter azotocaptans,* and *Azospirillum lipoferum* on corn plant grown under greenhouse conditions. Microbial Ecol. 51:326-335.

Mertens, T., and D. Hess. 1984. Yield increases in spring wheat (*Triticum aestivum* L.) inoculated with *Azospirillum lipoferum* under greenhouse and field conditions of a temperate region. Plant Soil. 82:87-99.

Meyer, P.D., and G.W. Gee. 1999. Flux-based estimation of field capacity. J. Geotech. Geoenviron. Eng. 125:595-599.

Mia, M.A.B., and Z.H. Shamsuddin. 2009. Enhanced emergence and vigor seedling production of rice through plant growth promoting bacterial inoculation. Res. J. Seed Sci. 2:96-104.

Mia, M.A.B., Z.H. Shamsuddin, W. Zakaria, and M. Marziah. 1999. External and internal root colonization of *Azospirillum brasilense* on tissue-cultured banana plantlets. Proceedings of 8th Scientific Conference Electron Microscopy Society, (SCEMS'00), Genting Highlands, Pahang, Malaysia. p.173-174.

Micanovic, D., V. Zecevic, D. Urosevic, and D. Knezevic. 2006. Nitrogen content in the seedlings of wheat as parameter for selection on nitrogen fixation. Kragujevac. J. Sci. 28:159-164.

Minamisawa, K., K. Nishioka, T. Miyaki, B. Ye, T. Miyamoto, M. You, A. Saito, M. Saito, W. Barraquio, N. Teaumroong, T. Sein, and T. Sato. 2003. Anaerobic nitrogen-fixing consortia consisting of clostridia isolated from gramineous plants. Appl. Environ. Microbiol. 70:3096-3102.

Mishustin, E.N., and V.K. Shil'Nikova. 1971. Biological fixation of atmospheric nitrogen. MacMillan, London.

Miyasaka, N., D. Thuler, E. Floh, W. Handro, M. Toledo, S. Gagioti, and H. Barbosa. 2003. During stationary phase, *Beijerinckia derxii* shows nitrogenase activity concomitant with the release and accumulation of nitrogenated substances. Microbiol. Res. 158:308-315.

Montoya, J.P., M. Voss, P. Kahler, and D.G. Capone. 1996. A simple, high precision, high-sensitivity tracer assay for N_2 fixation. Appl. Environ. Microbiol. 62:986–993.

Mulongoy, K., M. Gueye, and D.S.C. Spenser. 1992. Biological nitrogen fixation and sustainability of tropical agriculture. Proceedings of the 4th International Conference of the AABNF. International Institute for Tropical Agriculture, Ibadan, Nigeria, 24-28 September 1990. John Wiley and Sons, Chichester, UK.

Munoz-Rojas, J., L.E. Fuentes-Ramirez, and J. Caballero-Mellado. 2005. Antagonism among *Gluconacetobacter diazotrophicus* strains in culture media and in endophytic association. FEMS Microbiol. Ecol. 54:57-66.

Muthukumarasamy, R., G. Revathi, S. Seshadri, and C. Lakshminarasimhan. 2002. *Gluconacetobacter diazotrophicus* (syn. *Acetobacter diazotrophicus*) a promising diazotrophic endophyte in tropics. Current Sci. 83:137-145.

Nath, D.J., R.N. Bhattacharjee, M.R. Devi, and S.R. Patgiri. 1997. Widespread occurrence of *Azospirillum* spp. in the North-Eastern region of India. Adv. Plant Sci. 10:189-194.

Neal, J.L., and R.I. Larson. 1976. Acetylene reduction by bacteria isolated from the rhizosphere of wheat. Soil Biol. Biochem. 8:151-155.

Negi, M., K. Sadasivam, and K. Tilak. 1987. A note on the effect of nonsymbiotic nitrogen fixers and organic wastes on yield and nitrogen uptake of barley. Biol.Wastes. 22:179-185.

Neyra, C.A., and J. Dobereiner. 1977. Nitrogen fixation in grasses. Adv. Agron. 29:1-39.

Nguyen, T.H., I.R. Kennedy, and R.J. Roughley. 2002. The response of field-grown rice to inoculation with a multi-strain biofertilizer in the Hanoi district, Vietnam. In: I.R. Kennedy, and A.T.M.A. Choudhury, editors, Biofertilisers in action. RIRDC, Canberra, AU. p.37-44.

Nohrstedt, H.Ö. 1983. Natural formation of ethylene in forest soils and methods to correct results given by the acetylene-reduction assay. Soil Biol. Biochem. 15:281-286.

Nohrstedt, H.Ö. 1985. Non-symbiotic nitrogen fixation in the topsoil of some forest stands in central Sweden. Can. J. Forest Res. 15:715-722.

Novoa, R., and R.S. Loomis. 1981. Nitrogen and plant production. Plant Soil. 58:177-204.

Okon, Y., and Y. Kapulnik. 1986. Development and function of Azospirillum inoculated roots. Plant Soil 90:3-16.

Omar, S.A., and M.H. Abd-Alla. 1992. Effect of pesticides on growth, respiration and nitrogenase activity of *Azotobacter* and *Azosirillum.* World J. Microbiol. Biotechnol. 8:326-328.

Ortiz-Castro, R., H.A. Contreras-Cornejo, L. Macias-Rodriguez and J. Lopez-Bucio. 2009. The role of microbial signals in plant growth and development. Plant Signal Behav. 4:701-712.

Othman, A.A., W.M. Amer, M. Fayez, and N.A. Hegazi. 2004. Rhizosheath of Sinai desert plants is a potential repository for associative diazotrophs. Microbiol. Res. 159:285-293.

O'Toole, P.O., and R. Knowles. 1973. Efficiency of acetylene reduction (nitrogen fixation) in soil: effect of type and concentration of available carbohydrate. Soil Biol. Biochem. 5:789-797.

Pace, N. 1997. A molecular view of microbial diversity and the biosphere. Sci. 276:734-740.

Parker, C. 1957. Non-symbiotic nitrogen-fixing bacteria in soil III. Total nitrogen changes in a field soil. J Soil Sci. 8:48-59.

Patriquin, D.G., J. Dobereiner, and D.K. Jain. 1983. Sites and processes of association between diazotrophs and grasses. Can. J. Microbiol. 29:900-915.

Patterson, R.F., and M.A. Litt. 1989. Webster's Dictionary and Thesauraus. PSI and Associates, Miami, FL. p.41.

Paul, E.A., and F.E. Clark. 1996. Soil microbiology and biochemistry. 2nd Edition. Academic Press, California, USA.

Pedraza, R.O.. 2008. Recent advances in nitrogen-fixing acetic acid bacteria. Int. J. Food Microbiol. 125:25-35.

Penot, I., N. Berges, C. Guiguene, and J. Fages. 1992. Characterization of *Azospirillum* associated with maize (*Zea mays* L.) in France using biochemical tests and plasmid profiles. Can. J. Microbiol. 38:798-803.

Peoples, M.B., and J.A. Baldock. 2001. Nitrogen dynamics of pastures: nitrogen fixation inputs, the impact of legumes on soil nitrogen fertility, and the contributions of fixed nitrogen to Australian farming systems. Aust. J. Exp. Agric. 41:327-346.

Pereyra, M.A., F.M. Ballesteros, C.M. Creus, R.J. Sueldo, and C.A. Barassi. 2009. Seedlings growth promotion by *Azospirillum brasilense* under normal and drought conditions remains unaltered in Tebuconazole-treated wheat seeds. Europe. J. Soil Biol. 45:20-27.

Petrini, O. 1991. Fungal endophytes of tree leaves. In: J. Andrews, and S.S. Hirano, editors, Microbial ecology of leaves. Spring Verlag, New York, USA. p.179-197.

Phipps, P.M. 1984. Soybean and peanut seed treatment: new development and needs. Plant Dis. 68:76-77.

Plazinski, J., and B.G. Rolfe. 1985. Influence of *Azospirillum* strains on the nodulation of clovers by *Rhizobium* strains. App. Environ. Microbiol. 49:984-989.

Postgate, J. 1971. Biochemical and physiological studies with free-living, nitrogen-fixing bacteria. Plant Soil. 35:551-559.

Postgate, J. 1998. Nitrogen fixation. Cambridge University Press, Cambridge, UK.

Rai, S.N., and A.C. Gaur. 1982. Nitrogen fixation by *Azospirillum* spp. and effect of *Azospirillum lipoferum* on the yield and N-uptake of wheat crop. Plant Soil. 69:233-238.

Rai, S.N. and A.C. Gaur. 1988. Characterization of *Azotobacter* spp. and effect of *Azotobacter* and *Azospirillum* as inoculant on the yield and nitrogen-uptake of wheat crop. Plant Soil. 109:131-134.

Raj, N.S., G. Chaluvaraju, K.N. Amruthesh, H.S. Shetty, M.S. Reddy, and J.W. Kloepper. 2003. Induction of growth promotion and resistance against downy mildew on pearl millet (*Pennisetum glacum*) by rhizobacteria. Plant Dis. 87:380-384.

Rao, V., P. Jenna, and T. Adhya. 1987. Inoculation of rice with nitrogen-fixing bacteria – problems and perspectives. Biol. Fert. Soils. 4:21-26.

Rao, K.V.B., and P.B.B.N. Charyulu. 2005. Evaluation of effect of inoculation of *Azospirillum* on the yield of *Setaria italic* (L.). African J. Biotechnol. 4:989-995.

Rawluk, C.D.L., G.J. Racz, and C.A. Grant. 2000. Uptake of foliar or soil application of ^{15}N-labelled urea solution at anthesis and its effect on wheat grain yield and protein. Can. J. Plant Sci. 80:331-334.

Reed, S., C. Cleveland, and A. Townsend. 2007a. Controls over leaf litter and soil nitrogen fixation in two lowland tropical rain forests. Biotropica. 39:585-592.

Reed, S., T. Seastedt, C. Mann, K. Suding, A. Townsend, and K. Cherwin. 2007b. Phosphorus fertilization stimulates nitrogen fixation and increases inorganic nitrogen concentrations in a restored prairie. Appl. Soil Ecol. 36:238-242.

Reeves, T.G., S.R. Waddington, I. Ortiz-Monasterio, M. Banziger, and K. Cassady. 2002. Removing nutritional limits to maize and wheat production: a developing country perspective. In: I. Kennedy, and A.T.M.A. Choudhury, editors, Biofertilisers in action. RIRDC, Canberra, AU. p.11–36.

Reinhold, B., and T. Hurek. 1989. Location of diazotrophs in the root interior with special attention to the kallar grass association. In: F. Skinner et al., editors, Nitrogen fixation with non-legumes. Kluwer Academic Publishers. Dordrecht, The Netherlands. p.199-208.

Reichardt, K., G. Hardason, F. Zapata, C. Kirda, and S.K.A. Danso. 1987. Site variability effects on field measurement of symbiotic nitrogen fixation using the ^{15}N isotopic dilution method. Soil Biol. Biochem. 19:405-409.

Rennie, R.J. 1980. Isotope dilution as a measure of dinitrogen fixation by *Azospirillum brasilense* associated with maize. Can. J. Bot. 58:21-24.

Rennie, R.J., J.R. de Freitas, A.P. Ruschel, and P.V. Vose. 1983. ^{15}N isotope dilution to quantify dinitrogen (N_2) fixation associated with Canadian and Brazilian wheat. Can. J. Bot. 61:1667-1671.

Rennie, R.J., and S. Dubetz. 1984. Effect of fungicides and herbicides on the nodulation and N_2 fixation in soybean fields lacking indigenous *Rhizobium japonicum.* Agron. J. 76:451-454.

Rennie, R.J., and D.A. Rennie. 1983. Techniques for quantifying N_2 fixation in association with non-legumes under field and greenhouse conditions. Can. J. Microbiol. 29:1022-1035.

Rennie, R.J., and J.B. Thomas. 1987. ^{15}N-determined effect of inoculation with N_2 fixing bacteria on nitrogen assimilation in Western Canadian wheats. Plant Soil. 110:213-223.

Rice, W., and E. Paul. 1972. The organisms and biological processes involved in asymbiotic nitrogen fixation in waterlogged soil amended with straw. Can. J. Microbiol. 18:715-723.

Riggs, P.J., M.K. Chelius, A.L. Iniguez, S.M. Kaeppler, and E.W. Triplett. 2001. Enhanced maize productivity by inoculation with diazotrophic bacteria. Aust. J. Plant Physiol. 28:829-836.

Rippka, R., J. Deruelles, J. Waterbruy, M. Herdman, and R. Stanier. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111:1-61.

Roesch, L., F. Camargo, F. Bento, and E. Triplett. 2008. Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. Plant Soil. 302:91-104.

Roger, R.A., and J.K. Ladha. 1992. Biological N_2 fixation in wetland rice fields: estimation and contribution to nitrogen balance. Plant Soil. 141:41-55.

Roper, M.M. 1983. Field measurements of nitrogenase activity in soils amended with wheat straw. Aust. J. Agric. Res. 34:725-739.

Roper, M.M. 1985. Straw decomposition and nitrogenase activity (C_2H_2 reduction): effects of soil moisture and temperature. Soil Biol. Biochem. 17:65-71.

Roper, M.M., and D.M. Halsall. 1986. Use of products of straw decomposition by N_2-fixing (C_2H_2-reducing) populations of bacteria in three soils from wheat-growing areas. Aust. J. Agric. Res. 37:1-9.

Roper, M.M., and J.K. Ladha. 1995. Biological N_2 fixation by heterotrophic and phototrophic bacteria in association with straw. Plant Soil. 174:211-224.

Roper, M.M., G. Marschke, and N. Smith. 1989. Nitrogenase activity (C_2H_2 reduction) in soils following wheat straw retention: effects of straw management. Aust. J. Agric. Res. 40:241-253.

Roper, M.M., J. Turpin, and J. Thompson. 1994. Nitrogenase activity (C_2H_2 reduction) by free-living bacteria in soil in a long-term tillage and stubble management experiment on a vertisol. Soil Biol. Biochem. 26:1087-1091.

Roy, R.N., R.V. Misra, and A. Montanez. 2002. Decreasing reliance on mineral nitrogen – yet more food. J. Human Environ. 31:177-183.

Ruschel, A., Y. Henis, and E. Salati. 1975. Nitrogen ^{15}N tracing of N_2-fixation with soil grown sugarcane seedlings. Soil Biol. Biochem. 5:83-89.

Russow, R., M. Veste, and F. Böhme. 2005. A natural ^{15}N approach to determine the biological fixation of atmospheric nitrogen by biological soil crusts of the Negev Desert. Rapid Commun. Mass Spec. 19:3451-3456.

Rychert, R., J. Skujinš, D. Sorensen, and D. Porcella. 1978. Nitrogen fixation by lichens and free-living microorganisms in deserts. US/IBP Synthesis Series. 9:20-30.

Sachdev, D., H.G. Chaudhari, V.M. Kasture, D.D. Dhavale, and B.A. Chopade. 2009. Isolation and characterization of indole acetic acid (IAA) producing *Klebsiella pneumoniae* strains from rhizosphere of wheat (*Triticum aestivum*) and their effect on plant growth. Indian J. Exp. Biol. 47:993-1000.

Saharan, B.S., and V. Nehra. 2011. Plant growth promoting rhizobacteria: a critical review. Life Sci. Med. Res. Vol 2011:LSMR-21

Santa, O.R.D., R.F. Hernandez, G.L.M. Alvarez, P. Ronzelli Jr., and C.R. Soccol. 2004. *Azospirillum* sp. inoculation in wheat, barley and oats seeds greenhouse experiments. Braz. Archibes. Biol. Technol. 47:843-850.

Saikia, S., and V. Jain. 2007. Biological nitrogen fixation with non-legumes: An achievable target or a dogma? Current Sci. 92:317-322.

Sain, P., and F. Broadbent. 1977. Decomposition of rice straw in soils affected by some management factors. J. Environ. Qual. 6:96-100.

Salantur, A., A. Ozturk, and S. Akten. 2006. Growth and yield response of spring wheat (*Triticum aestivum* L.) to inoculation with rhizobacteria. Plant Soil Environ. 5:111-118.

Saralov, A., and T. Babanazarov. 1983. Characteristics of the microflora and nitrogen fixation in the takyr soils of the rice fields in the Karakapak ASSR. Microbiol. 51:682-686.

Saskatchewan Agriculture, Food and Rural Revitalization. 2010. Guide To Crop Protection

Schank, S., and R. Smith. 1984. Status and evaluation of associative grass-bacteria N_2-fixing systems in Florida. Proceedings, Soil and Crop Sci. Soc. FL. 43:120-123.

Schimel, J. 1995. Ecosystem consequences of microbial diversity and community structure. In: F.S. Chapin, and C. Korner, editors , Artic and alpine diversity: patterns, causes, and ecosystem consequences. Springer, New York, USA. p.241–254.

Sexstone, A., N. Revsbech, T. Parkin, and J. Tiedje. 1985. Direct measurement of oxygen profile and denitrification rate in soil aggregates. Soil Sci. Soc. Am. J. 49:645-651.

Shabaev, V.P. 1986. The effect of cropping and fertilizer nitrogen rates on nitrogen balance in soil. Plant Soil. 91:249-256.

Sheng, X.F., and W.Y. Huang. 2001. Physiological characteristics of strain NBT of silicate bacterium. Acta. Pedological. Senica. 38:569-574.

Shearer, G., and D. Kohl. 1988. Natural ^{15}N abundance as a method of estimating the contribution of biologically fixed nitrogen to N_2-fixing systems: potential for non-legumes. Plant Soil. 110:317-327.

Shearman, R., W. Pedersen, R. Klucas, and E. Kinbacher. 1979. Nitrogen fixation associated with ‘Park’ Kentucky bluegrass (*Poa pratensis* L.). Can. J. Microbiol. 25:1197-1200.

Shin, S.J., J.H. Han, E.J.B. Manning, and M.T. Collins. 2007. Rapid and reliable method for quantification of *Mycobacterium paratuberculosis* by use of ob BACTEC MGIT 960 system. J. Clinical Microbiol. 45:1941-1948.

Shrestha, R.K., and J.K. Ladha. 1996. Genotypic variation in promotion of rice dinitrogen fixation as determined by nitrogen-15-dilution. Soil Sci. Soc. Am. J. 60:1815-1821.

Sierra, J., and P. Renault. 1996. Respiratory activity and oxygen distribution in natural aggregates in relation to anaerobiosis. Soil Sci. Soc. Am. J. 60:1428-1438.

Sinclair, J.B., and P.A. Backman. 1989. Compendium of soybean diseases. 3rd Edition. APS Press, St. Paul, MN. p.24-27.

Six, J., K. Paustian, E.T. Elliott, and C. Combrink. 2000. Soil structure and organic matter: 1. Distriubution of aggregate-size classes and aggregate-associated carbon. Soil Sci. Soc. Am. J. 64:681-689.

Six, J., G. Guggenberger, K. Paustian, L. Haumaier, E.T. Elliott, and W. Zech. 2001. Sources and composition of soil organic matter fractions between and within soil aggregates. Eur. J. Soil Sci. 52:607-618.

Smith, R.L., J.H. Bouton, S.C. Schank, R.H. Quesenberry, M.E. Tyler, M.H. Gaskins, and R.C. Littell. 1976. Nitrogen fixation in grasses inoculated with *Spirillum lipoferum*. Sci. 193:1003-1005.

Smith, R.S., M.A. Ellis, and R.E. Smith. 1981. Effect of *Rhizobium japonicum* inoculant rates on soybean nodulation in a tropical soil. Agron. J. 73:505-508.

Smith, V.H. 1992. Effects of nitrogen: phosphorus supply ratios on nitrogen fixation in agricultural and pastoral ecosystems. Biogeochem. 18:19-35.

Soleimanzadeh, H., D. Habibi, M.R. Ardakani, F. Paknejad, and F. Rejali. 2010. Response of sunflower (*Helianthus annuus* L.) to inoculation with *Azotobacter* under different nitrogen levels. American-Eurasian J. Agric. Environ. Sci. 7:265-268.

Somado, E.A., and R.F. Kuehne. 2006. Appraisal of the ^{15}N-isotope dilution and ^{15}N natural abundance methods for quantifying nitrogen fixation by flood-tolerant green manure legumes. Afr. J. Biotechnol. 5:1210-1214.

Somasegaran, P. 1985. Inoculant production with diluted liquid cultures of *Rhizobium* spp. and autoclaved peat: evaluation of diluents, *Rhizobium* spp., peats, sterility requirements, storage, and plant effectiveness. Appl. Environ. Microbiol. 50:398-405.

Somers, E., and J. Vanderleyden. 2004. Rhizosphere bacterial signaling: a love parade beneath our feet. Crit. Rev. Microbiol. 30:205-240.

Soon, Y.K., and M.A. Arshad. 2002. Comparison of the decomposition and N and P mineralization of canola, pea and wheat residues. Biol. Fertil. Soil. 36:10-17.

Sriskandarojah, S., I.R. Kennedy, D. Yu, and Y.T. Tchan. 1993. Effects of plant growth regulators on acetylene-reducing associations between *Azospirillum brasilense* and wheat. Plant Soil. 153:165-178.

Steenhoudt, O., and J. Vanderleyden. 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol. Rev. 24:487-506.

Stewart, W. 1980. Systems involving blue-gree algae (cyanobacteria). In: F. Bergersen, editor, Methods for evaluating biological nitrogen fixation. John Wiley and Sons, Chichester, UK. p.583-635.

Steyn, P.L., and C.C. Delwiche. 1970. Nitrogen fixation by nonsymbiotic microorganisms in some California soils. Enviro. Sci. Technol. 4:1122-1128.

Stoltzfus, J., and F. de Bruijn. 2000. Evaluating diazotrophy, diversity, and endophytic colonization ability of bacteria isolated from surface-sterilized rice. In: J. Ladha, and P. Reddy, editors, The quest for nitrogen fixation in rice. Proceedings of the Third Working Group Meeting on Assessing Opportunities for Nitrogen Fixation in Rice. 9-12 August 1999. Laguna, Philippines, IRRI. p.63-91.

Stotzky, G. 1997. Soil as environment for microbial life. In: J. van Elsas et al., editors, Modern soil microbiology. Dekker, New York, USA. p.1-20.

Striganova, B.R., T.D. Pantosh-Derimova, and A.V. Tiunov. 1993. Comparative estimation of nitrogen fixation activity in the intestine of various species of earthworms. Biol. Bull. 2:257-263.

Strong, W.M., J. Harbison, R.G.H. Nielsen, B.D. Hall, and E.K. Best. 1986. Nitrogen availability in a Darling Downs soil following cereal, oilseed and grain legume crops. I. Soil nitrogen accumulation. Aust. J. Exp. Agric. 26:347-351.

Sturz, A.V., B.R. Christie, and J. Nowale 2000. Bacterial endophytes: potential role in developing sustainable systems of crop production. Crit. Rev. Plant Sci. 19:1-30.

Subbarao, G.V., B. Tomohiro, K. Masahiro, I. Osamu, H. Samejima, H.Y. Wang, S.J. Pearse, S. Gopalakrishnan, K. Nakahara, A.K.M.Z. Hossain, H. Tsujimoto, and W.L. Berry. 2007. Can biological nitrification inhibition (BNI) genes from perennial *Leymous racemosus* (Triticeae) combat nitrification in wheat farming? Plant Soil. 299:55-64.

Sukiman, H.I., and P.B. New. 1990. Relationships between root colonization and initial adsorption of *Azospirillum* to plant roots. Mirob. Ecol. 20:65-74.

Sullivan, P. 2001. Alternative soil amendments. Appropriate Technology Transfer for Rural Areas, NCAT. http://attra.neat.org/attra-pub/PDF/altsoil.pdf

Sumner, M.E. 1990. Crop responses to *Azospirillum* inoculation. Adv. Soil Sci. 12:53-123.

Suslow, T.V. 1982. Role of root-colonizing bacteria in plant growth. In: M. Mount, and G. Lacy, editors, Phytopathogenic prokaryotes. Academic Press, New York, USA. p.186-223.

Swędrzyńska, D., and A. Sawicka. 2001. Effect of inoculation on population numbers of *Azospirillum* bacteria under winter wheat, oat, and maize. Polish J. Environ. Studies. 1:21-25.

Tchan, Y. 1988. Some aspects of non-rhizobial diazotrophs: their past and their future. In: W. Murrell, and I. Kennedy, editors,. Microbiology in action. Research Studies Press/Wiley, Chichester, UK, p.193-207.

Turnbull, G.A., J.A.W. Morgan, J.M. Whipps and J.R. Saunders. 2001. The role of bacterial motility in the survival and spread of *Pseudomonas fluorescens* in soil and in the attachment of colonization of wheat roots. FEMS Microbiol. Ecol. 36:21-31.

Turner, G.L., and A.H. Gibson. 1980. Measurement of nitrogen fixation by indirect means. In: F.J. Bergersen, editor, Methods for evaluating biological nitrogen fixation. John Wiley and Sons, Chichester, UK. p.111-138.

Umali-Garcia, M., D.H. Hubbell, M.H. Gaskins, and F.B. Dazzo. 1980. Association of *Azospirillum* with grass roots. Appl. Env. Microbiol. 39:219-226.

Unkovich, M.J., and J. Baldock. 2008. Measurement of asymbiotic N_2 fixation in Australian agriculture. Soil Biol. Biochem. 1:1-7.

Unkovich, M.J., J.S. Pate, and J. Hamblin. 1994. The nitrogen economy of broadacre lupin in southwest Australia. Aust. J. Agric. Res. 45:149-164.

Urquiaga, S., K.H.S. Cruz, and R.M. Boddey. 1992. Contribution of nitrogen fixation to sugar cane: Nitrogen-15 and nitrogen-balance estimates. Soil Sci. Soc. Am. J. 56:105-114.

Vallis, I. 1973. Sampling for soil nitrogen changes in large areas of grazed pastures. Commun. Soil Sci. Plant Anal. 4:163-170.

van Berkum, P., and B.B. Bohlool. 1980. Evaluation of nitrogen fixation by bacteria in association with roots o tropical grasses. Microbiologic. Rev. 44:491-517.

Vande Broek, A., and J. Vanderleyden. 1995. The genetics of the *Azospirillum*-plant root association. Crit. Rev. Plant Sci. 14:445-466.

van der Burgt, G.J.H.M., and B.G.H. Timmermans. 2009. Fusarium in wheat. The effect of soil fertility strategies and nitrogen levels on mycotoxins and seedling blight. Louis Bolk Instituut. www.orgprints.org

van der Lee, G., B. de Winder, W. Bouten, and A. Tietema. 1999. Anoxic microsites in Douglas fir litter. Soil Biol. Biochem. 31:1295-1301.

van Elsas, J.D., and C.E. Heijenen. 1990. Methods for the introduction of bacteria into soil: a review. Biol Fertil Soils. 10:127-133.

van Elsas, J.D., A.F. Kijkstra, J.M. Govaert, and J.A. van Veen. 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Microbial. Ecol. 38:151-160.

van Elsas, J.D., and L.S. van Overbeek. 1993. Bacterial responses to soil stimuli. In: S. Kjelleberg, editor, Starvation in bacteria. Plenum Press, New York.

Van Loon, L.C.. 2007. Plant responses to plant growth-promoting bacteria. Eur. J. Plant Pathol. 119:243-254.

Vendan, R.T., and M. Thangajaru. 2007. Development and standardization of cyst based liquid formulation of *Azospirillum* bioinoculant. Acta. Microbiol. Immunol. Hung. 54:167–177.

Ventura, W., and I. Watanabe. 1983. ^{15}N dilution of accessing the contribution of nitrogen fixation to rice plant. Soil Sci. Plant Nutr. 29:123-131.

Villora, G., D.A. Moreno, and L. Romero. 2004. Crop quality under adverse conditions: Importance of determining the nutritional status. Product. Pract. Quality Assess. Food Crops. 2:59-78.

Vincent, J. 1984. Potential for enhancing biological nitrogen fixation. In: P. Jose, and S. Blixt, editors, Crop breeding: a contemporay basis. Pergamon Press, Oxford, UK. p.185-215.

Walley, F. 2005. Nitrogen mineralization: What's happening in your soil? http://www.ssca.ca/conference/conference2005/Walley.pdf

Walker, T.S., H.P. Bais, E. Grotewold, and J.M. Vivanco. 2003. Root exudation and rhizosphere biology. Plant Physiol. 132:44-51.

Wani, S., S. Chandrapalaih, and P. Dart. 1985. Responses of pearl millet cultivars to inoculation with nitrogen-fixing bacteria. Exp. Agric. 21:175-182.

Wani, P.A., A. Zaidi, A.A. Khan, and M.S. Khan. 2005. Effect of phorate on phosphate solubilization and indole acetic acid releasing potentials of rhizospheric microorganisms. Annals Plant Protect. Sci. 13:139-144.

Weber, O.B., V.L.D. Baldani, K.R.S. Teixeira, G. Kirchhof, J.I. Baldani, and J. Döbereiner. 1999. Isolation and characterization of diazotrophic bacteria from banana and pineapple plants. Plant Soil. 210:103-113.

Weier, K.L. 1980. Nitrogen-fixation associated with grasses. Tropical Grasslands. 14:194-201.

Weier, K.L., P.A. Pittaway and J.H. Wildin. 1995. Role of N_2-fixation in the sustainability of the ponded grass pasture system. Soil Biol. Biochem. 27:441-445.

Weller, D.M. 1988. Biological control of soil borne plant pathogens in the rhizosphere with bacteria. Ann. Rev. Phytopathol. 26:279-407.

Werner, D., R. Berggold, D. Jaeger, A. Krotzky, H. Papen, S. Schenk, and H. Thierfelder. 1989. Plant, microbial and soil factors, determining nitrogen fixation in the rhizosphere. Z. Pflanz. Bodenkunde.. 152:231-236.

Whipps, J. 2001. Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot.52: 487-511.

Wilson, D. 1995. Endophyte-the evolution of a term, and clarification of its use and definition. Oikos 73:274-276.

Wissuwa, M., M. Mazzola, and C. Pickard. 2009. Novel approaches in plant breeding for rhizosphere-related traits. Plant Soil. 321:409-430.

Witty, J.F. 1979. Acetylene reduction assay can overestimate nitrogen-fixation in soil. Soil Biol. Biochem. 11:209-210.

Witty, J.F., and J.M. Day. 1978. Use of $^{15}N_2$ in evaluating asymbiotic N_2 fixation. In: Isotopes in biological dinitrogen fixation. Proceedings of an advisory group meeting, Vienna. 21-25 November 1977. IAEA. p.135-148.

Wood, C.C., N. Islam, R.J. Ritchie, and I.R. Kennedy. 2001. A simplified model for assessing critical parameters during associative $^{15}N_2$ fixation between *Azospirillum* and wheat. Aust. J. Plant Phys. 28:969-974.

Woolfolk, C.W., W.R. Raun, G.V. Johnson, W.E. Thomason, R.W. Mullen, K.J. Wynn, and K.W. Freeman. 2002. Influence of late-season foliar nitrogen applications on yield and grain nitrogen in winter wheat. Agron. J. 94:429-434.

Wu, P., G. Zhang, J.K. Ladha, S.R. McCouch, and N. Huang. 1995. Molecular-marker-facilitated investigation on the ability to stimulate N_2 fixation in the rhizosphere by irrigated rice plants. Theor. Appl. Genetic. 91:1177-1183.

Wuest, S.B., and K.G. Cassman. 1992. Fertilizer-nitrogen use efficiency of irrigated wheat: I. Uptake efficiency of preplant versus late-season application. Agron. J. 84:682-688.

Yanni, Y.G., and F.K. Abd El-Fattah. 1999. Towards integrated biofertilization management with free living and associative dinitrogen fixers for enhancing rice performance in the Nile delta. Symbiosis. 27:319-331.

Yanni, Y.G., R.Y. Rizk, F.K.A. El-Fattah, A. Squartini and V. Corich. 2001. The beneficial plant-growth promoting association of *Rhizobium leguminosarium* bv. Trifolii with rice roots. Aust. J. Plant Physiol. 28:845-870.

Young, I.M., and K. Ritz. 2000. Tillage, habitat space and function of soil microbes. Soil Tillage Res. 53:201-213.

Zhang, L., T. Hurek, and B. Reinhold-Hurek. 2007. A *nif*H-based oligonucleotide microarray for functional diagnostics of nitrogen-fixing microorganisms. Microbial Ecol. 53:456-470.

Zehr, J.P., B.D. Jenkins, S.M. Short, and G.F. Steward. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environ. Microbiol. 5:539–554.

Zimmer, W. 1988. The phytohormonal interactions between *Azospirillum* and wheat. Plant Soil. 110:239-247.

8. APPENDICES

8.1 Appendix A: Slope comparison equation

Equation A.1: Slope comparison equation

Table A.1 Slope comparison of recovered *Gluconacetobacter azotocaptans* CFU from wheat with three seed treatments. These values were obtained based on CFU at 0 to 72 h after inoculation for all seed treatments.

Seed Treatments	Slope A	Slope B	A-B	Std Err A	Std Err A^2	Std Err B	Std Err B^2	A^2+B^2	Sqr Root A^2+B^2	Z	*P* Value
Non-sterilized vs. Dividend® XL RTA®	-0.039	-0.028	-0.010	0.003	9.90E-06	0.004	1.4E-05	2.4E-05	0.005	-2.17	0.98
Dividend® XL RTA® vs. Sterilized	-0.028	-0.026	-0.002	0.004	1.36E-05	0.0037	1.4E-05	2.8E-05	0.005	-0.35	0.63
Non-sterilized vs. Sterilized	-0.039	-0.026	-0.012	0.003	9.90E-06	0.0037	1.4E-05	2.4E-05	0.005	-2.53	0.99

Table A.2 Slope comparison of recovered *Azospirillum lipoferum* CFU from wheat with three seed treatments. These values were obtained based on CFU at 0 to 72 h after inoculation for non-sterilized and Dividend® XL RTA® seed treatments, and 0 to 24 h after inoculation for the sterilized seed treatment.

Seed Treatments	Slope A	Slope B	A-B	Std Err A	Std Err A^2	Std Err B	Std Err B^2	A^2+B^2	Sqr Root A^2+B^2	Z	*P* Value
Non-sterilized vs. Dividend® XL RTA®	-0.002	-0.006	0.004	0.003	1.09E-05	0.003	1.2E-05	2.29E-05	0.00478	0.879	0.1897
Dividend® XL RTA® vs. Sterilized	-0.006	-0.036	0.029	0.003	1.19E-05	0.015	0.0002	0.0002	0.01505	1.964	0.0248
Non-sterilized vs. Sterilized	-0.002	-0.036	0.034	0.003	1.09E-05	0.015	0.0002	0.0002	0.01502	2.248	0.0123

$$Z = \frac{b_1 - b_2}{\sqrt{SEb_1^2 + SEb_2^2}}$$

Then use the website http://world-class-manufacturimg.com/Sigma/z.html to find *P* for calculated z use one tailed test.

8.2 Appendix B: Moisture holding capacity of growth chamber soil

Equation B.1 Moisture holding capacity of growth chamber soil

$$\text{MHC} = \frac{(\text{weight of wet soil} - \text{weight of dry soil})}{\text{weight of dry soil}} \text{ X } 100$$

The average total moisture holding capacity of the soil was found to be 27.9%.

The volume of soil was 2850 g pot^{-1}.

MHC (%)	Amount water needed to be added to 2850 g pot^{-1} soil
100	795.15 mL
80	636.12 mL
60	477.09 mL
40	318.06 mL

8.3 Appendix C: Determination of ^{15}N application for growth chamber pots

Equation C.1 Determination of ^{15}N application for growth chamber pots

Adding the equivalent of 5 kg N ha^{-1} of 10 atom% to each pot
Each pot is 0.0314 m^2 pot^{-1}

$$\frac{5 \text{ kg N ha}^{-1}}{0.0314 \text{ m}^2\text{pot}^{-1}} = \text{mg required pot}^{-1}$$

$$\text{mg N pot}^{-1} \times 45\% \text{ N in urea fertilizer} = \text{amount of 10 atom\% pot}^{-1}$$

8.4 Appendix D: Field studies site design

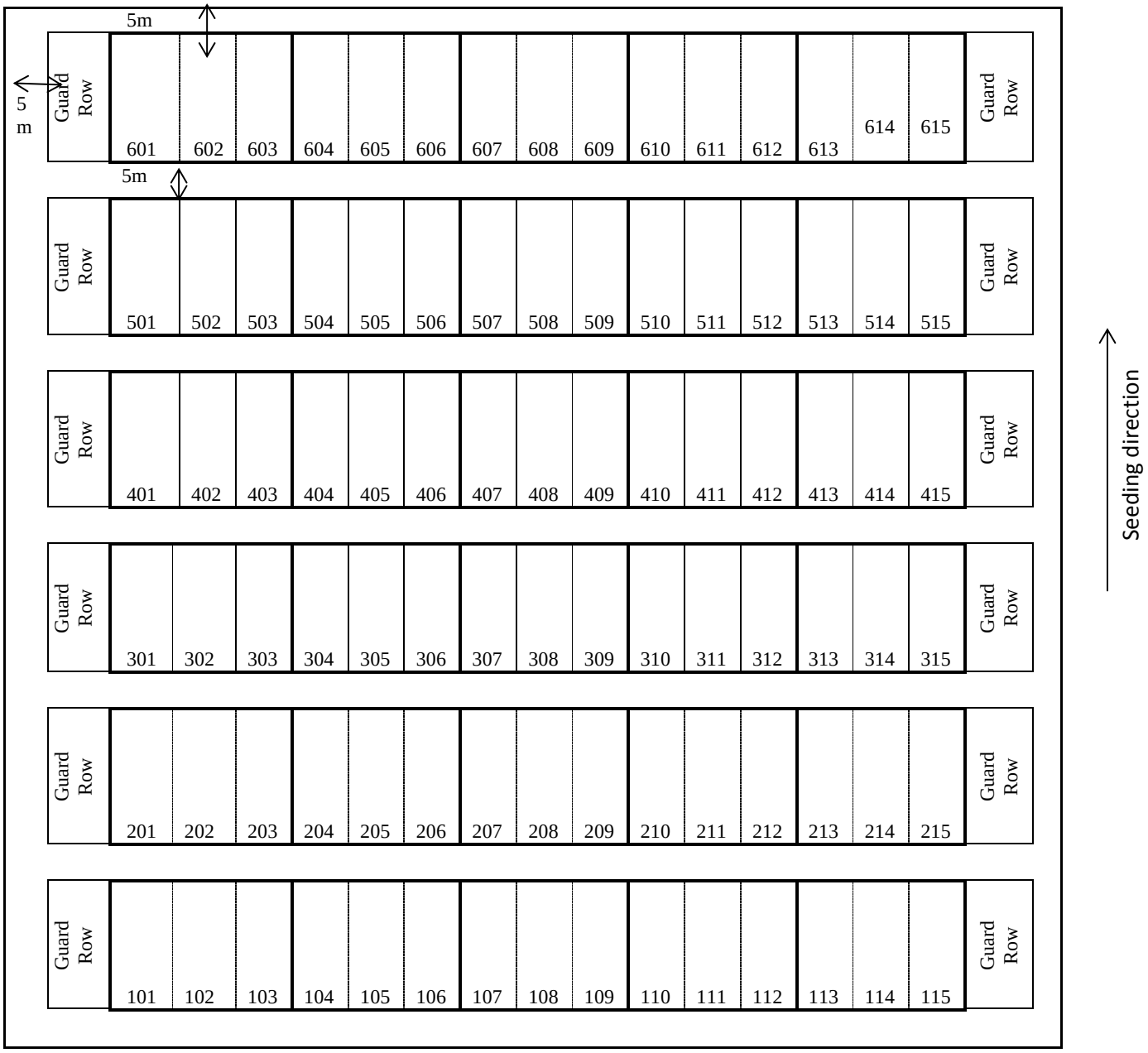


Figure D.1 Field plot design. Plots of 3 in each rep separated by the bold border are main effects (fertilizer applied at 0, 20, 40, 60 and 80 kg N ha^{-1}), and in each main effect were each of the sub-effects (seed treatments).

8.5 Appendix E: Field studies site design with applied microplots

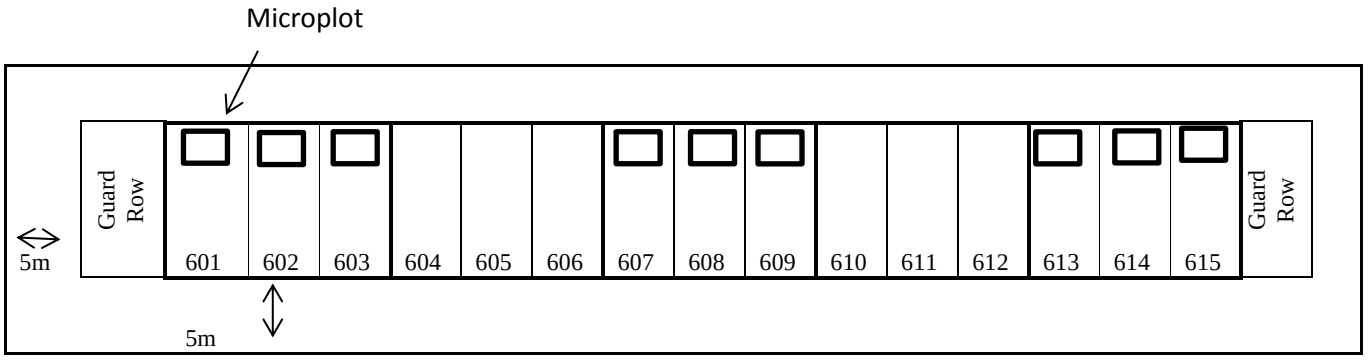


Figure E.1 Field plot design with microplots. Plots of 3 in each rep separated by the bold border are main effects (fertilizer applied at 0, 20, 40, 60 and 80 kg N ha^{-1}), and in each main effect were each of the sub-effects (seed treatments). Inside plots fertilized with 0, 40 and 80 kg N ha^{-1} was a microplot (1 m^2). Example of a replicate.

8.6 Appendix F: Determination of ^{15}N application for field experiment microplots

Equation F.1 Determination of ^{15}N application for field experiment microplots

At each site, adding ^{15}N fertilizer to 3 N treatments (0, 40, 80 kg N ha^{-1}) at 5 kg N ha^{-1}

$$3 \text{ N trt X } 3 \text{ organisms X } 6 \text{ reps } = 54 \text{ plots/site}$$

$$54 \text{ plots site}^{-1} \text{ X } 1 \text{ m}^2 \text{ microplots } = 54 \text{ m}^2 \text{ X } 1 \text{ ha } 10000\text{m}^2 = 0.0054 \text{ ha}$$

$$5 \text{ kg N ha}^{-1} = 5\text{kg X } 0.054 \text{ ha } = 0.027 \text{ kg N needed } = 27\text{g N}$$

Urea = $CO(NH_2)_2$
C=12
O=16
N=15 X 2=30
H=1 X 4=4

Total Molecular Weight = 62

=48.39% N in $CO(NH_2)_2$

$$\frac{27 \text{ g}}{0.484} = 55.79 \text{ g CO(NH2)2}$$

$$\frac{56 \text{ g}}{54 \text{ plots}} = 1.037 \text{ g CO(NH2)2 per plot}$$